

Computers for British academics

The provision of computers in British universities has been generous while the rest of the system has been starved. The government should put computers on an equal footing with other equipment.

How can a university system (the British) generally supposed to be on the brink of collapse from penury be advocating expenditure of £47.5 million on the provision of supercomputers as a service for academic research? This will be one reaction to the report published this week (see p. 569). Indeed, many readers of the report will be surprised to learn that British universities already have access to two machines in the supercomputer class and that, moreover, there is a dedicated data transmission network linking all British universities with each others' machines and the two supercomputers. The explanation is that since the early 1970s, when the organization called the Computer Board was established, computing facilities at British universities have been dealt with separately from the other costs of academic institutions, and generously as well. During the past few years, funds in excess of £30 million a year have passed through the Computer Board's hands. Although the board, like the University Grants Committee, is a creature of the Department of Education and Science, it seems to have escaped lightly in the general rush to cut. The result is that British universities have reasonably good access to computing facilities. That is as it should be.

It is also right and proper that plans should be made now to keep the network up to scratch. Last week's report is both persuasive and arresting. The chief recommendation is that a decision should be made now to order a more advanced Cray machine, to be installed by 1988, and that the interval should be used for improving the data network to handle the extra flow of data. There are also sensible schemes for installing parallel processing equipment locally. Then, the committee argues, a decision should be made not later than the end of the decade on the specification of a second supercomputer specifically for academic use. There is the strongest possible reason why the British government (which will ultimately have to pay) should accept the committee's argument. Grumbles from elsewhere in the university system that £47 million could be better spent in different ways should not be allowed to carry too much weight. Even a university system generally in decline should be allowed to do some things excellently.

That should be the immediate response to a generally sensible report. The weakness of the committee's argument is chiefly administrative. That there should be a peer-review procedure for deciding who should have the right to bid for time on such supercomputer capacity as there may be makes sense. The idea that there should be a second tier of assessment to decide which problems have solutions of practical utility is more dubious. But the new proposal that there should be an entirely new board (euphemism for committee) with exclusive responsibility for the machines that happen, just now, to be called supercomputers makes no sense at all. This is a recipe for setting up a new committee whenever the evolution of computer hardware throw

up a radical novel generation of machines.

The reason why this week's committee includes among its sponsors not merely the Computer Board but the Advisory Board for the Research Councils and the University Grants Committee, is that the sums of money involved are conspicuous. The Computer Board itself cannot hope to continue raising the funds it needs for general improvements and also to purchase supercomputers. But academic computers have come to stay, and like other scientific equipment will go on getting more powerful and (not as quickly) more expensive. The Computer Board itself should be recognized as an anomaly and subsumed within a larger organization. □

Worries about deficits

The failure of the US Congress to put the government's deficit to rights is serious for us all.

How much will the US deficit be in the financial year beginning on 1 October? \$200,000 million, the equivalent of \$700 for each man, woman, child and alien in the United States? Or 10 per cent above or below that figure? That is the untidy question the US government (the administration and the Congress in mutual cowardliness) has left for the rest of us to worry about. By now, the world knows that President Ronald Reagan rejected at the end of last month the plan of his party allies in the US Senate to save \$45,000 million by means of an oil import tax and the two-yearly staggering of social security revaluations, the Congress in panic settled for a mixture of cuts on budget spending that the appropriations committees may think it politically prudent to ignore.

Professional newspapers such as this must in prudence not stray too far outside their terms of reference. Liberally minded readers will acknowledge that concern for the workings of the educational system is proper, if only because that is what delivers recruits to the profession. Politically contentious though it may often be, even arms control is a permissible subject, partly because it has technical content, partly because research would come to an end if the cause of hard-headed arms control should fail. But why the US deficit? Because that too has technical content. And because the dice the US government is now tumbling could as certainly bring science and the enlightenment to a halt as an outright nuclear war.

Here is how the catastrophe may come about. For the past century, the United States has proved itself to be the most innovative and liberal of the mercantile economies of the modern world. The result is that people have become willing to lend money to US enterprises, knowing that in such an adaptable economy, the borrowers will probably make better use of the money than they could themselves. Moreover, they are usually confident that if things go wrong, they can hope to get their money back. Part of the reason for this confidence is that the US government historically has made only small demands on the US money system.

The big change is the deficit, at \$200,000 million roughly four per cent of the Gross National Product of the United States. The paradoxical situation has arisen in which foreigners finance a large chunk of industrial development, while the government in

Biological manuscripts

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Washington borrows its \$200,000 million a year largely from its own citizens and corporations. Interest rates are higher than they need be, given general confidence in the United States and the low rate of inflation over the past few years. The result is that the US dollar is artificially high compared with other currencies, which means that important sections of US industry, from agriculture to manufacturing, are competitively disadvantaged.

That the present circumstances are unstable is demonstrated by the fluctuations on the international currency markets. The threat to the US banking system occasioned by more than \$50,000 million of overseas loans is technically a separate issue, but one that could precipitate a loss of confidence in the dollar. Otherwise, there are two distinct ways in which the international community will deal with the deficit if the US government does nothing much about it: either people overseas will decline to keep on lending to the United States unless interest rates are further increased, in which case industrial production will further decline, the loss of confidence may go so far as to cause a flight from the dollar, in which case the currency will fall to where it should be, but corporations in the United States will be strapped for the cash they need to finance development. That is how the rest of the world sees the problem. In the United States, the prevailing sense, by contrast, is that trading partners are taking unfair advantage of the strong dollar, whence the widespread cry for protection from the rigours of free trade. The biggest danger now is that the Congress will listen more attentively to these voices than to the need to cut the deficit. The consequence, protectionism and counter-protectionism, could be just as serious as straightforward collapse. □

Action to follow

The US Food and Drug Administration plans to be more energetic. Time will tell if it succeeds.

Ms Margaret Heckler, Secretary of Health and Human Services in the US administration and, now that Mr James Watt has left the Department of the Interior, one of its most eager publicists, has embarked on a herculean task — that of preparing the Food and Drug Administration (FDA) for “the challenges of the twenty-first century”. More strictly, Ms Heckler has delegated the task to Dr Frank Young, director of the agency for the past year. Late last month, the two of them made public what the FDA calls *A plan for action*, a judicious blend of good intentions and more distant but even better aspirations. The temptation to make fun of this ingenuous document should, however, be repressed. Since the days during the Chinese cultural revolution when it was customary for Chinese public agencies to confess the errors of their past ways, and promise to do better in the future, there has been nothing like FDA’s vision of what it will become.

The starting point is tangible enough, the new procedures for the approval of new drug applications (NDA), published in final form in February. The intention is to simplify and accelerate the process of approving new drugs, partly by making FDA’s requirements of manufacturers more explicit, partly by admitting evidence gathered outside the United States in support of applications for generic drugs, for example. Along the same lines, a new set of regulations about Investigational New Drugs (IND) is about to make its appearance. Sensible proposals like these may not entirely meet the promise that the drug approval process may be shortened significantly from the present standard interval of 7–10 years, but they may at least prevent the further erosion of performance occasioned, among other things, by the increased sophistication of the testing procedures it is now possible (and prudent) to expect of applicants.

From that point on, the action plan becomes a good deal more fanciful. One proposal is that manufacturers submitting evidence in support of NDAs should be allowed to do so electronically, direct from their computer to another at FDA, thus doing away with the need to hire a truck to deliver the 100,000 pages of evidence which on the average accompany each application. The snag is that FDA has not yet equipped itself to receive these

electronic messages, and will be able to do so only when it can cheese-pare the funds from elsewhere in its budget. The scheme for deepening the range of expertise available to FDA by appointing at universities a number of FDA fellows who would divide their time between research and the giving of advice is similarly still only a sentence or two in the plan. But FDA’s proposals for further improving the process of data collection and analysis as part of its surveillance of drugs already in use should be largely a matter of good housekeeping, and thus well within its own control. □

Geostationary blues

Governments are perplexed about the diplomacy of geostationary satellites.

A WORLD Administrative Radio Conference (WARC) of the International Telecommunication Union (ITU) must rank with anything on the subject of Canada as the surest way for newspapers to lose readers. But responsible newspapers acknowledge that, damn it, from time to time, ITU’s WARCs have to be written about. The WARC now in session in Geneva, for example, is important: it will make assignments lasting a decade or more on radio frequencies and orbital positions for telecommunications satellites. Science writers have had to use all their ingenuity to make the subject comprehensible and, they fondly hope, interesting. And the biggest obstacles to their hopes are the names of the organization and of the conference and the technical problems involved.

The geostationary orbit, the circular path 36,000 kilometres above the Earth from which satellites appear to hover fixed over the same spot, is the hardest. The *Financial Times* made it sound quite cosy: “the most popular place for communications satellites . . . the ring in space which is home to about 80 operational communications satellites”. The *New York Times* disdained all technicalities and called it simply “the satellite belt”. How to brighten the name of the conference and the specialized agency of the United Nations which is running it? *Variety*’s “**Geneva Confab**” cannot be bettered, but neither can it be imitated. Other papers had to make do with phrases like “a six-week international gathering”. The *New York Times* did not mention the sponsoring organization.

Popularization is all very well but overdramatization is not. The need to make WARC interesting has pushed the newspapers into stating its purpose as unsnarling the “traffic jam in the heavens” or the “congestion over the Equator”. That is wrong, but the concept of a traffic jam is a poor metaphor. All the articles in which it appeared have then had to back away, explaining that there can be no physical pile-up and that the chances of collision in a path 265,000 kilometres in circumference are nil. Signal interference could be a problem, but even that is soluble with re-use of frequencies, higher frequencies and time-division multiplexing.

The real reason the conference was called is that the developing countries demanded it at the 1979 WARC. The United States, Britain and other industrial countries fought hard against it, but India insisted and, in the end, they had to give in. The developing countries demanded that they must have guaranteed access to places in orbit when they are ready, and so do not want the best positions to have been pre-empted by the industrialized countries. Equity in orbit is the problem, not congestion, and it will be tackled at two WARCs — this summer’s and the second session set for 1988. The United States is fighting advance reservations on the grounds that the practice wastes spectrum and discourages innovation. But it concedes that it has to do something to placate the developing world’s legitimate gripe about the industrialized world’s monopoly on the orbit. This grows out of ITU’s long-established policy of awarding all frequencies on a first-come, first-served basis. The poorer countries do not like that either. About 90 per cent of the usable frequencies in the radio spectrum are occupied by 10 per cent of the 150 member countries of the ITU. □

Gene therapy

NIH/FDA dispute likely to delay research

Washington

THE US National Institutes of Health (NIH) have reached an uneasy truce with the Food and Drug Administration (FDA) in a dispute over regulation of human gene therapy. NIH's Recombinant DNA Advisory Committee (RAC) has spent the past two years drawing up a list of "points to consider" in evaluating gene therapy proposals, but a recent attempt by FDA to muscle in on the act had threatened a further lengthy round of reviews before experimental treatments could commence. Experimental gene therapies are now expected to be ready for trials before the end of the year.

The first gene therapy proposals will probably entail treating either Lesch-Nyhan syndrome or adenosine deaminase deficiency, both rare inherited diseases, by infecting patients' bone marrow cells with a retrovirus carrying the gene the patient lacks; the cells would then be replaced in the patient. *Points to consider*, drafted by a group under LeRoy Walters of Georgetown University, has already been published for comment in the *Federal Register* and a final version was to have been approved in September. Two weeks ago, however, FDA exercised its new authority as the official "lead agency" for biotechnology regulation and proposed that guidelines on gene therapy or deliberate release of engineered micro-organisms should be reviewed by the new Biotechnology Science Board, a body that is to coordinate all federal agencies' interests in biotechnology. Meanwhile, FDA, as lead agency, would take control in areas where jurisdictions overlapped.

The Biotechnology Science Board has as yet no members, however, and it has not even been officially chartered. Faced with abandoning the past two years' work and having to start again, Walters' group has cancelled its September meeting.

The meeting at which FDA staged its coup attempt took place in the absence of NIH director James Wyngaarden. On learning of the FDA proposals, Wyngaarden met with FDA commissioner Frank Young (long rumoured as a future assistant secretary of health and unofficial "biotechnology czar") and secured an agreement that NIH would go ahead with *Points to consider* on the understanding that it applied only to NIH-funded research. The document will now once again be published for comment. As the first gene therapy proposals are likely to come from NIH-funded researchers, this measure should at least ensure that someone will read them when they arrive.

There are some points of substance in

the FDA/NIH dispute, besides the squabble over jurisdiction. RAC's meetings are held entirely in public, except when proprietary information is being discussed; FDA, in contrast, conducts its reviews in private. Wyngaarden plays down the importance of the dispute and says the confusion of roles has now been clarified: he points out that once bone marrow cells have been removed from the body and treated with a virus, they technically become "biologics" and so within FDA's purview. Historically, however, FDA has tended to turn a blind eye to *bona fide* researchers performing, for example, experimental diagnostic tests; it is not going to be so lax with gene therapy. Researchers will apparently have to seek approval for experiments from both NIH and FDA.

French science

Xenophobia out of fashion

THIS may not be an auspicious time to speak of an international stance in French scientific and technological relations — after President François Mitterrand has said "non" to star wars research, and after France has pulled out of European advanced fighter-plane project — but this is just what the French council of ministers was doing last week.

In a brief communiqué, the council (the French cabinet) said it wished to "intensify European cooperation", notably in relation to the Eureka project for new European high technology products, and would "reinforce our scientific and technological relations with the United States and Japan".

French research students are also to be encouraged to do their PhDs in collaboration with a foreign university, foreign researchers are to be given a better welcome in France than hitherto, and French scientific diplomatic representation abroad is to be reorganized, the communiqué said.

What this means in practice, however, is difficult to determine. The bid to improve research relations with the United States and Japan was "95 per cent exhortation", according to a French official, as it applied to industry, "over which we have little control", despite French industry's high degree of nationalization. However the intention was clear — to encourage French companies to learn from the Japanese and the Americans how to make a profit out of research: how to apply scientific know-how. For the feeling is that in certain sectors of the French economy, the government may have succeeded in putting researchers in touch with industry —

FDA officials say that is exactly the kind of over-regulation they are seeking to avoid. There also are criticisms of the way the NIH group under Walters, which is a subcommittee of RAC is constituted. One FDA official says that somatic cell therapy (the only sort contemplated in the near future) is analogous to many other medical procedures and so should be regulated similarly, by technical experts rather than by lawyers and bioethicists such as are found in Walters' group.

Points to consider describes the documentation needed to support a proposal for gene therapy: structure of the DNA construct, its purity, details of preparation, expression and animal studies; it also outlines the need for informed consent, measures to protect patient confidentiality and accurate information for news media. RAC is already considering one proposal that would allow it to bow out of areas where another agency has assumed responsibility for regulation; under this proposal, however, NIH would decide for themselves which areas to surrender.

Tim Beardsley

but there is some puzzlement in industry about what to do next.

In relation to universities, last week's communiqué may have more impact "as they take note of what government says". Jean-Pierre Chevènement, as a member of cabinet, has given the communiqué his backing, and must implement its recommendations. Thus it is likely that there will now be pressure on universities to design PhD courses in partial collaboration with foreign universities. How this might be done will be up to the universities, but the "habilitation" system, where universities must apply regularly for specific approvals to run postgraduate courses, would give the minister considerable power to influence the course of events.

Equally, it will be up to Chevènement and minister of research Hubert Curien to implement the recommendation that foreign researchers visiting France should be given better conditions of service. This means better grants for research students visiting France and fewer bureaucratic obstacles for more senior visiting scientists — despite the fact that last year all government-funded research positions became civil service posts.

As for French scientific diplomats, who are thicker on the ground in many countries than those of any other nation, there will be an attempt to "rationalize" their distribution and training, and, according to Paris officials, to cut costs. Many small countries enjoy the presence of a French scientific diplomat even though there is little research: such countries may lose their science counsellor, while larger countries may get more. Robert Walgate.

Ocean Drilling Project

Britain still seeks for membership

WITH six weeks to go before the deadline, Britain is still scrambling to find 40–50 per cent of the £2.5 million fee needed to resume membership in the Ocean Drilling Project (ODP). As the crunch nears, Dr John Bowman, secretary of the Natural Environment Research Council (NERC), says the drive to participate has intensified. But so far, the roughly £1 million needed to take Britain over the top has not materialized. Britain was a member of the previous United States-led Deep Sea Drilling Program, but has not joined ODP for lack of funds.

"There is no doubt anywhere", says Dr Bowman, "of the scientific merit of ODP." Geologist Joe Cann, closely connected with ODP since 1975, described the importance of the new phase of the project. During the drilling of new holes, data will be gathered by sophisticated logging instruments (see *Nature* 316, 8; 1985); the holes will also be used for long-term monitoring of continuing processes beneath the sea floor, rather than as mere sources of rock samples. One of the most significant of the planned ODP projects, according to Dr Cann, is to intercept the deposits formed at the submarine hot springs that spew zinc, iron and copper sulphides into the ocean.

Mr Ed Nickless of NERC claims that 100 per cent of the membership fee could follow from talks now going on between the Department of Energy (DOE) and the oil industry, but not everyone is as optimistic. DOE, according to Dr Cann, is interested only in funding the drilling of holes in British waters that can then provide stratigraphic data of immediate relevance to British oil interests. The oil industry, which would prefer to do its own exploratory drilling, supports ODP as a source of ideas, not drilled holes. But Dr Cann points to the number of strategic ideas gathered from previous ODP trips that have been turned into exploratory tools for the oil industry in an "impressively rapid time — 5–10 instead of the usual 20–30 year span".

Refusal to dig deep into pockets also results from competition. British Petroleum, says Cann, looks at its direct competitors, primarily Exxon, and sees no reason why it should fund its country's involvement in ODP when the US company makes no comparable sacrifice. Additional industry money, therefore, could well not become available without an arrangement about taxation that would allow both industry and government to save face.

Dr Bowman defends the government's position, saying the shortfall of £1 million is a bigger hurdle than it may seem, particularly to observers in the United States, Japan and France, countries "with a lot more money to spend on the physical sciences". Besides, he says, there are many reasons to fund scientific research other

than the data gathered. But this, according to Dr Cann, is the real problem with British funding for the physical sciences in general and ODP in particular.

One case in point is British involvement in Antarctic research. So that a strong presence can be maintained there, the budget of the British Antarctic Survey (BAS) has been doubled in three years, from £6 million in 1982 to £12.96 in 1985. The results, Dr Frank Laws, head of BAS, is quick to point out, are easy to find — more staff, building programmes, satellite communication, heavy-cargo operations and funding of university research.

Yet these large sums of money earmarked for Antarctica do not give BAS unusual independence from NERC, says Dr Bowman. BAS just happens to be the primary research group funded by NERC which is involved in Antarctica. Because of ODP's work in the Weddell Sea, moreover, Dr Bowman says that rechanneling some of the Antarctic funds towards ODP would be appropriate. That amount, according to Dr Laws, would be a "significant contribu-

tion" that would still "not seriously affect" BAS operations. By the same test, says Bowman, any sums taken from BAS would come nowhere near the elusive sum needed for British ODP membership.

Fiddling with budgets, says Dr Cann, can only hide the real problem. He claims that ODP decisions "have not been taken on any scientific basis at all," that "no real scientific presentation" has allowed, for example, "an analysis of the relative scientific importance of ODP and BAS". The compartmentalization and politicization of such budgetary decisions is a description supported by the Royal Society Report on Geophysics, which sees Britain often taking on the role of poor relation in international efforts such as ODP. "Somebody, somewhere", says Dr Cann, "should make an impartial judgement" about the relative importance of physical science research as well as about ODP.

If Britain fails to find the full membership fee by 1 October, efforts will continue to join at a later date. NERC has put the ODP fee as second in priority to university research in its requests to the Advisory Board for the Research Councils, which will adjudicate in late November or early December.

Elizabeth Collins

UK research

All change at research council

IN what may seem the nick of time, Britain's largest research council, the Science and Engineering Research Council (SERC), has found a new chairman. Professor E. J. W. ("Bill") Mitchell, head of



the Clarendon Laboratory at the University of Oxford, will begin a five-year stint on 1 October, only a month after his predecessor, Sir John Kingman, leaves to become vice-chancellor of the University of Bristol. At the same time, no fewer than six members of the 17-strong council will be replaced, at the end of their four-year terms, by newcomers.

Professor Mitchell is a solid-state physicist with a reputation as a forceful admi-

nistrator. His succession as chairman of SERC has been rumoured for some months. As a member of SERC since 1982, he will know of the unfinished business on the council's agenda. There is still no decision on the future of British collaboration with CERN, the high-energy physics laboratory at Geneva, while two important issues (support for astronomy and biotechnology) have been delegated to working parties that are likely to report only later in the year. The council's own corporate plan is still being worked on, while there is no certainty about the way in which funds will be divided between the five research councils next April.

The newcomers include Mr David Shore, technical director of APV Holdings, who will become chairman of SERC's engineering board; Dr Charles Reece, director of research and technology at ICI; Professor Malcolm Jeeves, the psychologist who is now vice-principal of St Andrew's University; Professor Brian Fender, vice-chancellor of Keele University and formerly director of the Institut Laue-Langevin, who will be chairman of SERC's science board; Professor Robert Wilson, professor of astronomy at University College, London; Professor Donald Perkins from the University of Oxford, a particle physicist who will become chairman of SERC's nuclear physics board; and Professor David Davies from the University of London, an optical fibre specialist. □

Supercomputing

Meeting British academic needs

A CENTRAL facility for advanced research computing is recommended this week by the report of a working party chaired by Professor A. Forty from the University of Warwick and commissioned by the Advisory Board for the Research Councils (ABRC), the Computer Board for Universities and Research Councils and the University Grants Committee (UGC).

The report proposes that the central installation should consist of the most powerful supercomputer available; the existing joint academic network (called Janet) should be enhanced for good communications; there should be a distributed system of other forms of advanced research computing to enhance local resources; and there should be a national organization to ensure effective use of resources, industrial and international collaboration and to stimulate new developments.

The cost of implementing the proposals will be about £47.5 million between 1986 and 1991. This includes £15 million for installation of the Cray X-MP multiprocessor array system at the Rutherford Appleton Laboratory, £8 million for high-speed lines and switches and £16.5 million for special purpose computers such as developments of ICL's DAP and GEC's GRID systems, which use distributed arrays of processors to perform operations in parallel. Running costs will be about £1 million a year. Purchase of a further supercomputer should be considered in 1988.

The report also proposes the machinery for running the facility: a board to advise ABRC, UGC and the Computer Board on advanced research computing needs and to award fellowships; and a peer review system to regulate use of facilities.

ABRC, UGC and the Computer Board must now decide whether they agree with the proposals and, if so, whether to allocate the money. ABRC is waiting for the individual research council's reactions to the report.

Recent advances in computing — vectorization and "parallel architecture" (simultaneous similar operations) — mean that the Cray 1 is 200 times faster than the VAX superminicomputer and 3,000 times faster and cheaper to use than the Atlas, the British supercomputer of the 1960s. Scientists in Britain currently have access to two supercomputers, the Cray 15 in London and the Cyber 205 in Manchester, linked by Janet. The London centre is now oversubscribed and Manchester is reaching saturation point. (There are also two DAPs in London and Edinburgh.) These supercomputers are not the most powerful models at present available.

The Forty report says the situation is different in other countries. In the United

States, the National Science Foundation programme started in 1984 involves the setting up of four supercomputer research centres ultimately to be linked by a national communications network. The government provides \$7–13 million a year to each centre and an equivalent sum is provided by industry, institutions and individual states.

In Japan, the seven main universities have a "special relationship" with one of the three major computer manufacturers (Hitachi, NEC and Fujitsu). Tokyo University, for example, has a Hitachi 5–810/20 supercomputer and Kyoto University a Fujitsu VP1100, both having replaced scalar machines at no extra cost. Other European countries such as France and West Germany are rapidly installing supercomputers and providing coordinated research programmes.

The new report's assessment of British needs is based on a questionnaire sent to

more than 400 individuals and scientific organizations and a detailed review of each subject provided by specialists in the field. The survey conclusively shows the need for a wide range of supercomputing facilities in astronomy, physics, earth sciences, engineering, biological and biomedical sciences, economics and information science.

Apart from the rapid understanding of many diverse scientific processes, the report emphasizes the use of supercomputers in industrial applications such as design and testing of pharmaceutical products using simulations, simulation and testing of aircraft engines and oil field modelling.

According to the report, there are compelling reasons for a coordinated and ambitious plan for the development of supercomputers in research in Britain. Haste is urged if Britain is to remain a world leader in the computer arena. Whether the bodies that commissioned the survey will agree, and if so, where the facility will be built and developed, remains to be seen.

Maxine Clarke

European training

Commission plan for mobility

Brussels

A FURTHER boost is to be given by the European Economic Community (EEC) to cooperation between European industry and academic institutions. The European Commission is advocating an 80 million ECU (about £44 million) four-year programme to stimulate on-the-job advanced training for students in companies in European countries other than their own. The scheme will also support exchanges between academics and those employed in new technology industries.

Later this year, the Commission hopes to persuade the ten ministers to agree to the four-year initial phase (1986–89) of the COMETT programme (Community in Education and Training for Technology).

Following in the steps of other Community initiatives such as ESPRIT, BRITE, the stimulation programme steered by the CODEST committee and the plan for transnational development of support in innovation and technology transfer, the programme will concentrate on new technologies, where it is felt that there is a particular need for more investment in training to keep up with rapidly changing technology.

The proposed programme would include exchange schemes between people in industry and universities as well as joint training projects between companies in different countries and the setting up of a European liaison network between university/industry training partnerships (UITPS).

Specifically, the Commission intends to start by offering some 2,000 six-month grants worth 4,000 ECU each for students

to train with a company in another Community country, with 3,000 places in 1988 and 5,000 in 1989.

A smaller number of grants (50 in 1987, to be doubled in 1988 and again in 1989) of 9,000 ECU would be available to university teaching staff willing to spend two or three terms in one year broadening their industrial experience in a company in another EEC country.

The Commission hopes there will be two kinds of joint training projects, one focusing on specific topics where skill is scarce and one designed to improve the flow of research results between industry and the universities. The Commission is prepared to pay 35 per cent of the cost up to a maximum of 500,000 ECU. It is also prepared to cover up to half the cost (a maximum of 400,000 ECU) for multilateral university/industry projects aimed at using new technologies in education and training itself. The COMETT team would also carry out a feasibility study for a European technological open university.

Some Community money would also be set aside (50,000 ECU a year) to cover half the running costs of promoting links between university/industry training partnerships, forming a Community-wide network coordinated by the Commission that would link up 40 UITPS in 1987 and 150 by 1989.

Finally, the Commission would also sponsor round-table meetings to bring industry and university together and would set up a database on cooperation, monitoring and analysis of Community trends and issues in advanced training.

Anna Lubinska

Space physics

NASA told to be more active

Washington

THE US National Research Council has told the National Aeronautics and Space Administration (NASA) to reverse the "long-term decline" in Solar System space physics, and has helpfully prescribed an ambitious series of projects to accomplish the task. The council's report* on the subject urges NASA to give highest priority to the International Solar Terrestrial Physics programme (ISTP), for which launches should start in 1989.

This influential support for ISTP should be music to the ears of NASA's partners in the project, Japan and the European Space Agency (ESA); three years ago, NASA infuriated European scientists by reneging on a promise to supply a satellite for the International Solar Polar Mission (now known as Ulysses). NASA officials now say there is "a reasonably good chance" that the agency will include funds to start work on ISTP in its budget request for fiscal year 1987 which NASA will finalize by 15 September.

The other two missions vying with ISTP for inclusion as a 1987 "new start" are

TOPEX (a satellite to measure ocean topography) and CRAF (Comet Rendezvous and Asteroid Flyby); the latest betting at NASA headquarters is that TOPEX and either CRAF or ISTP will be approved. In addition, the 1987 budget request is likely to include work on the mirror for AXAF, the Advanced X-Ray Astronomy Facility. By developing AXAF in stages, NASA hopes to avoid the embarrassing cost over-runs that plagued development of the Hubble Space Telescope.

The National Research Council Report, chaired by Stamatis M. Krimigis of the Applied Physics Laboratory at Johns Hopkins University, assumed a plausible (if hopeful) budget for Solar System space science of \$400 million by 1987 (in 1985 dollars); the current figure is \$300 million. NASA officials say the projects described in Krimigis's report are well justified and that it will be for Congress to decide how many will be funded.

Krimigis's report gives its blessing to the Upper Atmosphere Research Satellite, now being developed, and urges "immedi-

ate" funding for development of the Solar Optical Telescope, a 1.25-metre-aperture instrument that would form the basis of a solar observatory on the space station. It also wants to see a start on a long talked-about solar probe that would approach the Sun to within 4 solar radii; work should begin immediately on a heat shield for the mission in time for a possible launch in 1995. Looking further to the future, the committee gives its blessing to a solar polar orbiter that could be launched by the end of the century if a suitable propulsion system is developed.

Besides the highly visible major missions, the research council endorses an expansion of ground-based modelling and theoretical work in plasma physics; one goal is to provide an account of how disturbances on the Sun affect the heliosphere and thence the magnetospheres, ionospheres and upper atmospheres of the Earth and planets. There should also be a series of Explorer payloads, according to the committee, starting in the 1990s, to investigate Solar System space physics.

Besides being extremely colourful, the report is admirably specific in its recommendations. The committee's purpose might well be to avoid the fate of the 1980 Kennel Report on the same subject, on which the committee plaintively notes there has been "no significant progress" because of budget constraints.

Tim Beardsley

*An implementation plan for priorities in Solar-System space physics (Executive summary). Committee on Solar and Space Physics, National Research Council, 1985.

European research

EEC plans risk management

Brussels

THE European Commission has suggested to ministers of the European Economic Community (EEC) that 15 million ECU (European Currency Units, 1 ECU = £0.60) be provided over the next five years for research into the prevention, prediction and management of industrial accidents involving chemicals such as those that occurred in Seveso, Italy, in 1976 and last December in Bhopal, India.

Part of the new five-year 150 million ECU programme that also includes environmental protection and climatology, contracts on major technical hazards, to which the Community would contribute half the costs, will complement research already under way at the commission's own Joint Research Centre. These contracts will involve both industry and research institutes. It is hoped also to involve researchers from outside the EEC.

The aim will be to improve the scientific foundation of risk analysis by studying the physical and chemical phenomena of dispersion, runaway reactions and other causes of chemical accidents. There will also be an attempt to improve or replace hazardous processes, to improve safety technology, and to detect and mitigate high-risk situations. The Commission proposes that a start should be made on the transportation of bulk hazardous substances and on hazardous production processes.

The proposal would strengthen EEC

legislation sparked off by the Seveso disaster, when highly toxic dioxin was spewed accidentally over a north Italian town. The directive on the prevention of major industrial accidents came into force in 1981, but its implementation has posed difficulties for the member states.

In the environmental part of the research programme, for which 65 million ECU has been set aside, emphasis will be placed on health and ecological effects of pollution (particularly the deterioration of historic monuments), air, water and soil quality (including the impact of farming practices), waste, chemicals assessment (including the development of methods to avoid using vertebrates in toxicity tests) and noise. Emphasis will be put on advanced pollution abatement technology, in the hope that the outcome will also improve industrial competitiveness and employment prospects.

The Commission also intends to promote training in environmental sciences. Finally, 250 million ECU will be set aside for the second phase of a programme of long-term climate research. Work will continue on the physical basis of climate modelling, seasonal forecasting of European climates, climate sensitivity, effects of changes in atmospheric composition (via carbon dioxide and trace gases) and changes in land-surface properties, detection of climatic change and climatic impact on land, water, vegetation and marine resources.

Anna Lubinska

Polish protest (cont'd)

POLAND'S unofficial Social Committee for Learning, SKN (*Nature* 314, 123; 1985) has been quick to respond to the enactment, on 25 July, of the government-sponsored amendments to the 1982 Higher Education Act. A copy of SKN's irregular news bulletin *Servis informacyjny SKN*, dated 28 July, presents a reprint of the official announcement of the new law with the "tentative summary" of the effect of the changes, prepared by the Main Council for Science and Technology, the official representative body of the universities.

In the opinion of the Main Council, "without embarking on a major discussion of the motives and grounds for the proposed changes, they are clearly aimed at the creation of a legal framework for the direct and centralized direction of all matters relating to higher education in Poland, not only in questions of its organization but also in a wide range of questions of cadre policy and scientific research, not excluding legalized para-censorship".

The SKN bulletin also notes, however, that in the opinion of Deputy Jarema Maciszewski, who presented the bill to the Sejm: "The proposed changes do not contravene the idea of university self-government, but simply restore its purely academic character".

Vera Rich

Japanese psychiatry

Mounting pressure for reform

Tokyo

JAPAN'S mental health system is once more under fire. Representatives of the International Commission of Jurists (ICJ) and the newly formed International Commission of Health Professionals (ICHP) who came to Japan on a fact-finding mission in May published their first report last week. It makes disturbing reading.

The present "structure and function of the Japanese mental health service" is summed up as "conducive to inappropriate forms of care and serious human rights violations on a significant scale". As examples, the report lists "unacceptable conditions of overcrowding and poor nutrition which may lead to physical deterioration of patients and high rates of mortality; physical abuse of patients; exploitation of patient labour; unjustifiable detention; the inability of hospitalized patients to communicate with friends and family members outside the hospital or to receive visits under reasonable conditions".

That description may seem more fitting to a prison camp than a place of care for the sick but there is, regrettably, evidence that such conditions have existed in Japan. The report does not, however, mention specific cases; its avowed aim is to comment on the "overall system" rather than on individual occurrences.

Had specific cases been taken up, there is no doubt that the Utsunomiya hospital would have been first on the list. The hospital became the subject of major national scandal last year. It seems that violence and confinement were regularly used to subdue patients, mail was censored, meetings with outsiders prevented, patients forced into labour, unqualified staff employed, money embezzled and patients admitted and detained illegally.

Such at least were the more minor allegations made against the hospital staff; the serious allegation was that two patients were beaten to death, one for trying to escape. The hospital superintendent is now in prison but the case is far from cleared up. Independent psychiatric examinations after the scandal resulted in 40 per cent of the hospital patients being discharged: but there are accusations that a significant number of patients are still being unnecessarily detained.

Within the past few weeks, similar accusations have been made at another hospital, the Otaki hospital in Chiba prefecture. Once again there are allegations that a patient has been beaten to death by hospital staff. And two patients hospitalized after family quarrels and kept for long periods in locked wards have been judged sane by independent psychiatrists.

Despite the publicity given to these incidents, it would be quite wrong to think of

them as typical; most doctors and hospital staff are conscientious and see these abuses as "isolated exceptions". But what the ICJ/ICHP report makes clear is that the present Japanese legal system makes it all too easy for abuses to occur, however conscientious the average doctor may be — without reform there will continue to be "isolated exceptions". The major driv-



ing force behind the hospital abuses is undoubtedly greed. More than 80 per cent of psychiatric beds are in private mental hospitals and huge profits can be made. The temptation to cut patient care and hospital costs is great, especially as government supervision is minimal.

The report makes a number of specific recommendations for reform. Most notably, it is suggested that all cases of "involuntary hospitalization" be subject to independent review. As a recent government survey has shown, 93 per cent of inpatients are confined against their will (against just 5 per cent in the United Kingdom) and 80 per cent are confined under Article 33 of the Mental Health Act. This article allows the superintendent of a mental hospital to commit a patient indefinitely without the patient's consent and without the opinion of an independent psychiatrist provided the person responsible for the patient gives consent.

Clearly, as hospital profits require full beds, it is all too easy for a hospital superintendent to be tempted to misuse his powers. Stories have even been circulated of hospitals rounding up tramps from the streets of Tokyo in order to fill their wards; although such stories have never been substantiated, the fact that serious newspapers publish them at all suggests that public confidence in mental institutions is not high.

The report further suggests that an independent review tribunal be established, mental hospitals be regularly inspected and patients have right of access to the tribunal. At present, Article 38 of the Mental Health Act provides that "the administrator of the hospital may set necessary limits to the action of any in-

mate...". This extraordinarily wide power given to the hospital administrator has allowed censoring of mail, and refusal of visits and independent psychiatric assessment. Some patients have virtually been turned into prisoners without rights.

The report sees the general thrust of reform as towards the "community care" style of psychiatric treatment now espoused (if not adequately supported) in the West: that means short periods of hospitalization, provision of out-clinics attached to health centres, sheltered "half-way" houses, home visits by health workers, patient clubs, help in finding employment and so on. Such measures contrast starkly with the present Japanese system of large hospitals located in remote regions where land is cheap and long hospitalization periods. And while mental hospital populations are declining in the United States and Britain, those in Japan are increasing.

The report does not seek, however, to impose external standards of care on Japan and repeatedly stresses that changes need to be adapted "to meet the needs of Japanese society". Nor does the report paint a universally black picture. Recognition is given to psychiatrists, health officials and concerned citizens who are already actively trying to bring about change, and mention is made of innovative mental health programmes already in existence.

Given the clear aim of the report to be constructive, the response of the Japanese government has been very disappointing, to say the least. All the government has done is to question whether the report was truly the unanimous opinion of ICJ and to dispute the view, presented in the ICJ/ICHP report, that a previous critical report from the World Health Organization had not led to any changes in Japan's mental health policy. The first question was based purely on rumour and was quickly dismissed by ICJ. The second appears to be mere nitpicking. Not one of the major recommendations of the WHO report has been implemented. Given that it was published in 1968, astonishingly little progress has been made.

Hopes for change in the future are not bright. Considerable stigma is still attached to the mentally ill and it is unlikely that public support for reform will grow rapidly. A number of hospitals have introduced reforms but few of them are in the private sector, and without government backing, change will be slow. The major impetus for reform seems to come from foreign critics. The problems of Japan's hospitals are, at least, becoming internationally known. One of Japan's foremost campaigners for reform of the Mental Health Act, lawyer Etsuro Totsuka, spoke for the first time at a meeting of the British reform group MIND a few weeks ago. The ICJ/ICHP report will be submitted to the UN Subcommittee on Human Rights in the near future. **Alun Anderson**

Catastrophism still unexplained

SIR—Despite Napier and Clube's frequent (and frequently changing) statements on the nature of terrestrial catastrophism and possible extraterrestrial causes, they were not the first to point out that the Sun's motion in the Galaxy might be the causative agent, as stated by John Maddox (*Nature* **315**, 627; 1985). At least one earlier suggestion of this idea was a quite excellent paper by K.A. Innanen and colleagues entitled, "The interaction of the spiral density wave and the Sun's galactic orbit"¹ which appeared in 1978. That paper noted and displayed a figure showing the coincidence between galactic plane crossings and the boundaries between geological periods on the Earth.

Also, if I remember correctly, Napier and Clube² suggested that the important mechanism would be the Sun's passage through galactic spiral arms which happens every 250 Myr or so, as opposed to galactic plane crossings which occur every 33 Myr.

The periodicity in the terrestrial extinction record is certainly in grave doubt at this time. But I suggest that if there is any real periodicity it is most likely associated with a period of which we are already aware, that is the Sun's epicyclic z-motion about the galactic plane, rather than any of the artificial constructs we have seen suggested, such as the hypothetical death star or planet X. However, the problem of finding the extinction mechanism which might be associated with the Sun's epicyclic motion still remains unsolved.

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1. Innanen, K.A., Patrick, A.T. & Duley, W.W. *Astrophys. Space Sci.* **57**, 511–515 (1978).

2. Napier, W.M. & Clube, S.V.M. *Nature* **282**, 455–459 (1979).

Conference costs

SIR—It cannot be good that British scientists are unable to attend conferences in their own field held in their own country, a situation that now prevails.

American colleagues report to me that they come to conferences in the United Kingdom in the hope of meeting their British counterparts, only to find that apart from invited speakers, British academics, both staff and graduate students, are largely missing.

The cause is simple: *money*. Conference fees are now enormous (to pay presumably for the air fares of the overseas speakers), and there was even an institution which ran a "hundredth birthday" symposium from which its own staff were excluded unless they paid a large fee! At the same time, in order to stay afloat, the universities that host the conferences are

charging "economic" (meaning swinging) rates for accommodation and meals, while they and other publicly funded institutions are unable to reimburse anything but the smallest sums for attendance at conferences. (My own institution now pays a maximum of £40 a year, barely the train fare to London, and this by national standards is generous.) Erosion in the real value of salaries makes it difficult, particularly for younger staff with mortgages and children, to find the several hundred pounds that a conference may cost out of their own funds. British scientists are thus deprived of the international contacts that they should have, and of much needed opportunities to keep abreast of their fields.

Remedies are not so simple, but here are two suggestions: as all universities are levying enormous accommodation charges on each others' staff, they are in effect taxing their own staff for carrying out a necessary part of their work. An immediate convention should be set up by the Committee of Vice-Chancellors and Principals allowing British academics to be charged for accommodation at cost when attending a meeting at another university in this country. Second, the Royal Society should consider extending its system of grants for attendance at conferences overseas to include attendance at conferences in the United Kingdom.

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Value-free science

SIR—The idea of value-free science has been defended by two of your correspondents over the past several months, Bernard D. Davis (*Nature* **311**, 294; 1984 and **315**, 176; 1985) and M. Hammerton (**313**, 343 and **315**, 536; 1985). Unfortunately, they have not satisfactorily answered the points raised by Mark Diesendorf (**313**, 92 and **314**, 666; 1985).

Davis's illustration of the risk of fallout from nuclear weapons provides an excellent illustration of the different ways in which values are embodied in science.

Research related to nuclear weapons and fallout comes overwhelmingly from the governments of nuclear weapons states. That means that these governments influence the direction of scientific development. After all, values are involved in choosing a particular facet of the world to study and about which to formulate knowledge.

Knowledge about nuclear weapons and fallout is selectively useful to the governments of nuclear weapons states because they are the ones who can hire experts to apply it. Community groups do not have much use for nuclear science. Scientific knowledge is value-laden because it is selectively useful to different social groups.

Knowledge about hazards from fallout has often been used to justify continued nuclear weapons testing, ignoring the fact that the "benefits" of testing accrue to governments and militaries while the hazards literally fall on many people who obtain no benefit at all. Much scientific knowledge is value-laden because it serves to justify policies and practices of certain groups. Another example is the setting of "acceptable" levels of exposure to radiation: acceptable to whom?

In the past, defenders of nuclear technology have often adopted the hypothesis that there is a threshold beneath which radiation exposure poses no risk to health. This illustrates how the content of scientific knowledge can embody values.

These and other points are elaborated and illustrated in my study *The Bias of Science*.

It is futile to try to argue away or exorcise values from science, since this only obscures the role of the social context of science. More appropriate is the aim of making the values open and apparent, so that the direction and use of science can be a subject for public debate.

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Paul Rosbaud

SIR—The Houghton Mifflin Company will publish in 1986 a book about Dr Paul Rosbaud, scientific adviser to Springer Verlag in Berlin until 1945. After the Second World War, Rosbaud became associated with various scientific publishing activities in Britain until he died in 1963. Copies of letters, papers and so on associated with Rosbaud would be deeply appreciated by the undersigned. Particularly welcome would be any reminiscences about Rosbaud before 1946.

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Faith in God

SIR—In his review of Hugh Montefiore's book *The Probability of God* (*Nature* **315**, 353; 1985), John Maddox seems to imply that religious belief is almost some kind of moral and/or mental inadequacy. I would say rather that belief in God—any god—must ultimately be irrational, for it involves a step of faith. If belief were to be rational, that is, if the existence of God could be proved, then it would be meaningless, for surely a god whose existence can be proved by the activities of man is no god at all.

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Towards the quantum computer?

The Turing machine, an abstract computer conceived half a century ago as a test of what might be computable, has been overtaken by an idealized quantum computer, which also has drawbacks.

THE notion of the quantum computer means different things to different people. At one extreme are the hardware engineers, the people who behave (successfully) as if for every physical phenomenon there is at least one practical device. IBM's recent disappointments with Josephson junctions apart, these people know in their bones that the quantization of magnetic flux, as within closed loops of superconducting material, will one day be the basis of a memory storage element. The other extreme is the conceptual, typified by those who worry over the correspondence between the elementary units in which information is handled in computers and the quanta of the physical variables that characterize real physical systems.

A whole string of more substantial questions obviously suggest themselves. To what extent, for example, is the accuracy of a computer calculation limited by the deterministic uncertainty of quantum mechanics? Whatever the care with which signals are processed so as to encode a memory cell with either the digit zero or unity, the result cannot be entirely certain while the uncertainty principle remains in being. This is not a practical difficulty for the time being, while the scale of the most tightly-packed electronic components is measured in some hundreds of interatomic distances, but in due course the uncertainty principle must determine the limits to which it will make sense to carry very large-scale integration.

Meanwhile, but for a different purpose, Dr D. Deutsch from the Department of Astrophysics at the University of Oxford (*Proc. R. Soc. A* **400**, 97; 1985) has given what seems to be a way of describing the properties of a quantum computer which, among other things, has the virtue of showing the physical nature of the computing process. Quantum computers, it appears, are potentially more powerful in important ways than even that computational paradigm known as a Turing machine.

A Turing machine (first described by the late Dr Alan Turing nearly half a century ago) is, it will be recalled, a stripped-down computer, consisting only of an indefinitely long memory tape and a device (which would now be called a processor) whose state at any instant depends both on its previous state and on the most recently read item of information on the tape. On the assumption that the informa-

tion on the tape includes what would now be called the program as well as the input data, the pedagogical (some would say "heuristic") value of the Turing machine is to demonstrate the power or the limitations of the computing process, depending on one's point of view. Turing's purpose, in 1936, was to assert that such a machine, however rudimentary it might seem, could compute anything generally held to be computable. Deutsch, to be fair to J. Church (*Am. J. Math.* **58**, 435; 1936) calls this the Church-Turing principle.

Put like this, the principle sounds like a tautology. Deutsch argues that the tautology conceals a physical reality. The output datum of a computation will be a function of the input datum, so that the question of what may be held to be computable boils down to specifying the class of functions that can be realized by a Turing machine. Deutsch points out in passing that all that such a machine can do in practice is to make a map of the set of all the integers on itself, which is not exactly the same as defining the functional relationship between input and output (a point of some importance when so many people are interested in chaos, where a small change of an input variable may mean a very large change in the corresponding value of the output). More tellingly, Deutsch goes on to argue that the truth about the class of computable functions, or of conceivable algorithms, is that they are physically realizable. That mathematics is the handmaiden of physics is not an accident. And so Deutsch puts the "Church-Turing principle" in the form "Every finitely realizable physical system can be perfectly simulated by a universal model computing machine operating by finite means".

What kind of machine is that? Luckily, the formalism is not difficult, and can be put in words. The machine has a memory, which may in principle be infinite, but only one bit is involved at any step. There is a processor whose internal (quantum) state represents the progress of the computation. And there is an input/output tape which can move only one step (forwards or backwards) at a time, and whose position is an observable (in the sense of quantum mechanics). The evolution of the system as a whole from one step in the computation to the next is determined (as in ordinary wave mechanics) by the operation of some unitary operator on the state vector of the system lying within some appropriately catalogued Hilbert space. Although the dimen-

sionality of the problem will be dominated ordinarily by the number of memory cells, contributing that many powers of two, the problem is simplified because the elements of the unitary transformation matrix are themselves simplified by the requirement that the tape should move by only one step at a time, and that only one memory cell should be involved. One computer will differ from another in the structure of the unitary transforming matrix.

The formalism may be describable in words but the details show that the theory hangs together. Deutsch shows that a quantum computer (as defined) can carry out any computation of which a Turing machine is capable but that, because the elementary unitary transformation can mix together quantum states in ways that have no classical analogues, the universal quantum computing engine can yield results beyond the ken of classical machines.

For one thing, a quantum computer should be capable of generating true random numbers and not simulations thereof. (Proud custodians of new hardware who are convinced that their classical machines will generate random numbers for them had better read the 178 pages of *The Art of Computer Programming* Vol.2, in which D.E. Knuth explains why the programs purporting to give random numbers at the push of a button cannot be trusted.) Deutsch gives two sample programs. He goes on to show how to write a program (for a still non-existent quantum machine) to verify Bell's inequality about the essentially quantum character of quantum correlations. There are even a dozen lines of Algol 62 which simulate the Einstein-Podolski-Rosen experiment.

The interesting part of the argument concerns the interaction of the quantum computer with the outside world. To make this non-existent machine function, it is necessary that its initial state should be "prepared", in the sense of quantum mechanics, to be in some well-defined state, which is the same thing as saying that it has zero entropy. Deutsch himself points out that the third law of thermodynamics would make such a state inaccessible in finite time, goes on to say that the evolution in time of a small part of the Universe (such as a quantum computer) may be determined by a non-unitary transforming matrix and declares that it is of practical as well as of philosophical importance to learn what the rules are. Nobody will quarrel with that.

John Maddox

Molecular genetics

Introns continue to amaze

from Andy Flavell*

IN 1977 Joe Sambrook wrote a *News and Views* article in *Nature* entitled "Adenovirus Amazes at Cold Spring Harbor" in which he described the first experiments demonstrating RNA splicing¹. Since then we have learned that most eukaryotic and some prokaryotic genes contain intervening sequences (introns). Time has mellowed the initial astonishment but as we become accustomed to each new finding in this field, another fresh observation startles us out of our complacency. We have learned that some introns can remove themselves from precursor RNAs without recourse to proteins², others encode proteins which aid this process³ and many form circular excision products, some with strange 'lariat' structures^{4,5}. Those who thought that the flow of startling observations would diminish will have have to reconsider in the light of two sets of results, one of which is reported on page 641 of this issue⁶.

A big problem for molecular evolutionists has been the question of why genes have evolved in this manner⁷. One major school has suggested that introns have existed as long as recognizable genes^{8,10} and initially represented the junctions between small units of genetic information. Long precursor RNAs could self-splice to create assortments of these units, successful combinations being rewarded in the normal Darwinian manner. There were early attempts to argue that introns were vestigial mobile elements which had been transposed into genes¹¹⁻¹³ but no evidence emerged to support this notion. Recent results on yeast mitochondrial introns may, however, provide hard evidence that introns are far from passive sequences, with some perhaps able to ensure their survival by transposition to new locations^{6,14,15}.

Michel and Lang now demonstrate sequence homology between certain fungal mitochondrial messenger RNA introns and the reverse transcriptase genes of retroviruses⁶. Reverse transcriptase is the key enzyme in the retroviral life cycle; it converts the viral RNA into DNA, which becomes incorporated into the host genome. Michel and Lang have compared the predicted amino-acid sequences of the long open reading frames of four class II introns of fungal mitochondria with those of the highly conserved regions in retroviral polymerase genes. (Fungal mitochondrial introns are divided into two classes based on the presence of one of two sets of short sequence blocks that are

thought to determine their secondary structure in RNA^{16,17}). These regions are conserved between the *pol* genes of all known vertebrate retroviruses and retrovirus-related elements in yeast, plants and insects^{18,19}. The new work shows that each of the homologous segments that unite these introns are related to a corresponding conserved segment of the retroviral *pol* genes. The order of segments is conserved with a few differences in gap size between them. Lastly, the ten diagnostic predicted amino acids, which are virtually all present in *pol* genes, are similarly conserved in the class II open reading frames. It is thus likely that these open reading frames encode proteins that are related to reverse transcriptase.

What is known about the gene products of these class II open reading frames? The answer is quite a lot and none of it points to their encoding reverse transcriptase. The *a1* intron of the *oxi-3* gene (one of the four open reading frames) encodes a 'maturase', which has been elegantly demonstrated by Slonimski and coworkers to be necessary *in vivo* for the splicing out of the intron that encodes it²⁰.

It seems unlikely that a DNA reverse transcript of an intron participates in the removal of that intron from a precursor mRNA; it is more likely that the open reading frame gene product possesses both reverse transcriptase and maturase activity? There is some evidence to suggest that the maturase activity is not critical to the splicing event. Class I and class II introns are probably all spliced by a mechanism closely related to that of the self-splicing ribosomal RNA introns of *Tetrahymena*^{21,22}. It is thought that maturases aid the alignment of precursor RNAs, which then splice auto-catalytically^{6,21,22}.

Other nuclear genes have been identified in yeast that are necessary for splicing of mitochondrial introns in this fungus. A dominant mutation (probably a mis-sense point mutation) in one of these, *NAM-2*, cures a splicing deficiency resulting from the inactivation of the *b4* maturase (which is required for splicing of the *b4* intron of the *cob* gene containing it and also of intron *a4* of the *oxi-3* gene²³). The *NAM-2* mutant gene requires a gene product of the region containing exon 4 and intron *a4* for this action. These data show that maturases participate in splicing at some stage, but under some conditions a particular maturase is dispensable and there is an independent requirement for other gene product(s). They do not show what enzymic activity maturases possess. To complicate the picture further, if the wild-type *nam* allele is totally inactivated the mitochondrial genome is annihilated,

forming cytoplasmic *petite* mutants²³.

Michel and Lang argue that maturase function may be an accessory activity, regulating the level of the intron gene product, the major function of which is the maintenance of such introns by dissemination through the mitochondrial genome. Seen in this way, the fungal mitochondrial genome is an environment being exploited by an autoregulated class of mobile genetic elements. This is perhaps taking speculation a bit too far on the sole basis of amino-acid homologies. What is obviously required is the demonstration of reverse transcriptase activity in yeast mitochondria and, one hopes, from *in vitro* constructs containing these class II maturase genes. It will also be important to demonstrate transposition of class II introns, if possible. The authors cite two pieces of circumstantial evidence implying the presence of reverse transcriptase activity in fungal mitochondria — the occasional perfect excision of some *Saccharomyces cerevisiae* introns (presumably by gene conversions by a complementary DNA copy of the spliced mRNA), and the existence of a mitochondrial plasmid in *Podospora anserina* that is identical to one of the four introns they present. These arguments are persuasive but not conclusive.

If the authors are correct, these are the first identified reverse transcriptase genes of cellular rather than viral origin. It has been proposed that retroviruses evolved from mobile DNA elements that picked up an ancestral reverse transcriptase gene while wandering around the genome^{24,25}. A cornerstone of this theory is the cellular reverse transcriptase gene, which has been conspicuous by its absence from the literature till now. Perhaps some maturases are the progenitors of modern reverse transcriptases.

Are all introns explicable as mobile, self-splicing elements? Probably not; the class I and II introns are rare except in fungal mitochondria, an exception being the *Tetrahymena* 26S rRNA intron. Other introns fall into two classes, the nuclear mRNA introns and the transfer RNA introns. Neither of these possesses genes that affect their transposition, although it is always possible to invoke a small number of yet uncloned trans-acting genes. Both classes furthermore are excised by mechanisms very different from the class I and II introns, although mRNA splicing does create circular lariat intron excision intermediates that are reminiscent of the perfect circles created from class I/II excised introns^{3,4}. Nevertheless, the possibility that introns are relics of ancient mobile elements must still be considered. By this hypothesis the large majority of introns have long since evolved away from a migrating lifestyle.

Another possibility is that the class II maturase-containing introns are the descendants of the fortuitous incorporation of a reverse transcriptase gene by genomic rearrangement. The resulting construct

* I am grateful to Donal Hickey for communicating to me his ideas on intron evolution, in particular the possibility that introns are descendants of highly specialized retrotransposons. This will in part be presented as a letter in *Scientific Correspondence*.

would be able to ensure its survival by replicative transposition. This seems a more likely possibility: we know of many examples of DNA rearrangements and the success of mobile elements in eukaryotic genomes is similarly obvious. Of course, if the incorporated reverse transcriptase gene came from a retrovirus we are back at square one in our search for the ancestral gene.

How might an intron transpose using reverse transcriptase? For the intron to survive more than one cycle of retrotransposition, the ends of the element would have to correspond exactly to the region spliced out of the pre-mRNA (this is especially important for intron insertion in open reading frames). The splicing signals would need to be contained in the intron; this is the case for class I/II introns. The most attractive model employs the circular RNA molecules generated from introns after excision. It is easy to imagine reverse transcriptase converting these into a double-stranded DNA circle that would presumably be cleaved where the ends of the intron meet, and inserted into the acceptor site. One problem with this model is that the circles lose a few nucleotides from their 5' ends during circularization²⁶, and these would need to be restored upon integration to preserve the intron intact. One attraction of this model is that DNA circles are used in retrovirus proviral integration, thus to some extent unifying the two mechanisms.

Is there any evolutionary evidence for intron insertion in characterized gene families? The mammalian serine proteases have been thoroughly studied in this respect²⁷. There is strong circumstantial evidence supporting the insertion of introns into the chymotrypsin, elastase, urokinase and human tissue plasminogen activator genes. But these are nuclear mRNA introns and the reservations mentioned that these are potential mobile self-splicing elements must be considered.

One well-characterized example of in-

tron insertion occurs in yeast mitochondria. The class I r1 intron of the 21S rRNA gene of *S. cerevisiae* mitochondria is optional²⁸ and several other yeast mitochondrial introns are similarly heterogeneous. As alleles containing the r1 intron (ω^+ alleles) yield almost entirely ω^+ progeny when crossed with ω^- stocks, the intron seems to invade the ω^- 21S gene²⁹. The mechanism is thought to be a specialized form of gene conversion, with similarities to the yeast mating-type cassette switching at the *MAT* locus. Evidence for this comes from co-conversion of flanking genetic markers during $\omega^- \rightarrow \omega^+$ conversion and the presence of a double-stranded DNA break at the recipient site that is highly reminiscent of the y-z endo cut at the *MAT* locus³⁰. The exciting aspect of this mechanism is that the open reading

frame of the r1 intron is required for this process^{14,15}, showing that the intron encodes a gene product that catalyses its transposition. The r1 open reading frame shares homology with the maturase of the b2 intron of the yeast mitochondrial *cob* gene but not with the class II reverse transcriptase-related introns of Michel and Lang. However, unlike b2, the r1 gene product is not necessary for the splicing out of its own intron.

The connection between either the r1 type of intron or the class II intron of Michel and Lang and the maturase function is still unclear. Further study will not only clarify this issue but may tell us much about intron evolution. ■

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Polymer biochemistry

Radical breakdown of lignin

from Alistair Paterson and Knut Lundquist

ALTHOUGH certain fungi have no problem in degrading lignin, one of the major constituents of wood, biochemists have had a problem in explaining the mechanism by which a polymer with so many different inter-monomeric linkages is degraded. Intensive research has culminated in the discovery that the mechanism involves an enzymatic one-electron oxidation of lignin yielding aromatic radical cations that initiate cleavage of the side chains¹⁻³.

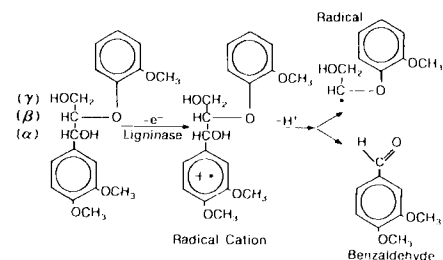
Lignin is synthesized by the addition of phenoxyl radicals, generated by dehydrogenation of the precursor *p*-hydroxycinnamyl alcohols, to the growing polymer chain. Most of the links in the resulting polymer are ether bonds between the central propanoid carbon (C β) of one monomer and the phenolic group of the next, referred to as β -aryl ether linkages, although nearly a dozen other bonds of carbon-carbon and ether type have been reported, giving lignin an irregular structure.

The most effective degraders of lignin are the white-rot fungi, of which the basidiomycete *Phanerochaete chrysosporium* is the best characterized. When growing in mycelial form in static liquid and solid culture, nutrient exhaustion initiates secondary metabolic or idiophasic activities in this fungus. At that time, it begins

to secrete small quantities of 'ligninase', a glycosylated haemoprotein, and to decompose lignin into CO₂. The ligninase was first described in 1983 by Ming Tien and T. Kent Kirk⁴ at the US Department of Agriculture Forest Products Laboratory in Madison, Wisconsin and by Michael Gold's group at Oregon Graduate Centre, Beaverton⁵. Initially, it was reported to require both hydrogen peroxide and molecular oxygen for activity and to degrade both a range of lignin model compounds and extracted native lignin.

The precise mechanism of ligninase activity has since been under detailed study, though limited by the small quantities of purified enzyme available. Now, Kirk and his group¹ and John Palmer and colleagues at Imperial College, London^{2,3} have shown that the initial reaction in the attack on lignin models is a one-electron oxidation that generates relatively stable aromatic radical cations in the lignin polymer. The haem-containing ligninase first reacts with hydrogen peroxide, producing a change in the ultraviolet absorption spectrum of the enzyme akin to that when an oxy-ferryl complex is formed in peroxidases. The enzyme is then able to oxidize lignin models in the presence or absence of oxygen; electron spin resonance studies of the oxidation of dimethoxybenzenes¹ and veratryl alcohol by ligninase yield spectra with signals that are consistent with the generation of the aromatic radicals.

This oxidation can be mimicked by either of two metal-centred oxidant and dimeric lignin model substrates containing an aryl ether linkage. One is tetraphenylporphyrinatoiron(III) in the presence of *tert*-butylhydroperoxide⁶, which is thought to model the oxy-ferryl complex of the monooxygenase cytochrome P-450



Suggested route of lignin depolymerization for a model compound of the β -aryl ether type.

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and peroxidases; the other is tris (phenanthroline) iron(III)³. The results suggest that the central feature of the 'ligninase' mechanism is the creation of the oxyferryl centre of high redox potential. Study of enzymatic attack on the lignin model substrate shows that a radical cation is formed by removal of an electron from the aromatic ring. The model compound then undergoes α - β alkyl cleavage with formation of a benzaldehyde and a radical, as shown in the figure. The latter can react with molecular oxygen, explaining the oxygen-18 labelled products observed in earlier reports.

Ligninase is clearly distinct from the metalloenzymes laccase and peroxidase, whose activity towards lignin model compounds has been thoroughly studied. Laccase is a copper-containing phenol oxidase that is produced by ligninolytic fungi but is also involved in the morphogenesis of non-ligninolytic basidiomycetes. This enzyme oxidizes substrates containing free phenolic hydroxyl groups, whereas ligninase is capable of oxidizing substrates with alkoxyl substituents on the ring; in the lignin polymer most of what were phenolic hydroxyls have been converted to the structural ether linkages. The oxidative changes in phenolic substrates — catalysed by ligninase or laccase — differ considerably from those produced in non-phenolic substrates by ligninase^{7,8}.

For some time, experimental evidence suggested that hydroxyl radicals, which are non-specific oxidants, mediate fungal degradation of lignin. But when Fenton's reagent, which chemically generates such radical species, is used to degrade lignin model compounds, the products differ from those of the enzymatic oxidation⁹. The specificity of the enzymatic reaction can be explained if its postulated oxyferryl centre has a lower redox potential than the hydroxyl radical but a higher potential than other peroxidases.

Once generated by the ligninase, the radical cations are themselves able to act as one-electron oxidants which can oxidize sites remote from the enzyme. Thus, despite the attention still being paid to extracellular proteins from ligninolytic *Phanerochaete chrysosporium*, there would seem to be no reason why ligninase (which is capable of cleaving many of the lignin intermonomeric linkages¹⁰) and laccase, together with the organism's oxidative cellulases (which have roles in reducing quinones¹¹ produced by oxidative lignin degradation and in generating the hydrogen peroxide¹² that is required to form the oxyferryl centres)

should not be able to catalyse all the lignin depolymerizations that have been observed up to now. □

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Astrophysics

New detectors for gravity waves

from James Hough

DIRECT detection of gravitational radiation remains one of the most challenging and difficult problems in experimental physics. Success in this field will be of considerable significance both for astronomy where new information about violent interactions in the universe may be obtained, and for physics where fundamental aspects of relativity theories may be checked. Indirect evidence for the existence of gravitational radiation has been provided by observations on the binary pulsar PSR 1913+16 and this provides added encouragement for the development of experiments in the field.

The detection of gravitational radiation relies on sensing the fractional strain or amplitude in space induced by the wave, through the effect on test masses, which may be essentially free or which may be coupled together mechanically. The resulting effective displacements of the test masses increase with their distance apart. Alternatively, the effect of the radiation on the phase of electromagnetic waves may be used. Radiation from possible sources ranges in frequency from 10^{-4} Hz for large black hole interactions and 10^{-3} Hz for fast binary stars to 10^3 Hz for stellar collapses (see table). To some extent the

frequency being searched determines the choice of detection method.

Detectors based on Earth look very promising for frequencies above several tens of Hertz, but are limited at low frequencies by the effects of seismic and man-made noise. At very low frequencies, one cycle per year or lower, pulsar timing is providing interesting limits on the gravitational wave background^{1,2}. In this case the pulsar is acting as a very stable clock, and the effect of gravitational radiation on the phase of its radio signals is being sought. A stable clock on Earth that forms the bases of a spacecraft tracking system can be used for higher frequencies, and Doppler tracking of interplanetary spacecraft has already allowed searches in the milliHertz range to be made at a gravitational-wave amplitude level of roughly 3×10^{-14} (ref. 3). The performance is limited by plasma scintillation affecting the radio signals. The significance of this may be reduced in future by using dual tracking frequencies, but better performance could be obtained by using optical frequency tracking. To avoid the effect of the Earth's atmosphere, however, the whole system would then need to be spaceborne.

In the long-term, the performance of

Some possible sources of gravitational radiation

Impulsive sources				
	Amplitude	Predominant frequencies	Rate	Detection method
Black hole events in galactic nuclei and quasars	$\sim 10^{-19} - 10^{-16}$	$10^{-1} - 10^{-4}$ Hz	< 50 per year	Doppler tracking initially then laser interferometer in space
Stellar collapse (at distance of Virgo Cluster)	$\sim 10^{-22} - 10^{-21}$	< 10^3 Hz	~ 1 per month	Cooled bar detectors, laser interferometers on Earth
Neutron star binary decay (out to 100 MPC, for example)	$\sim 10^{-22} - 10^{-21}$	$\sim 10 - 10^3$ Hz	$\sim$ few per year	Laser interferometers on Earth
Continuous sources				
	Frequency	Amplitude	Detection method (with integration for $\sim 10^6 - 10^7$ s)	
Pulsars				
For example, the Crab Nebula	60 Hz	< 10^{-25}	Laser interferometers on Earth	
Binary stars				
Iota Bootes	8.6×10^{-5} Hz	$\sim 5 \times 10^{-21}$	Laser interferometer in space	
Am CVn	2×10^{-3} Hz	$\sim 10^{-21}$	Laser interferometer in space	
Geminga ⁵	3×10^{-2} Hz	$\sim 10^{-19}$	Laser interferometer in space; 'Skyhook' detector	

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laser interferometry between test masses in satellites spaced a million kilometres or more apart looks very promising, with a potential sensitivity⁴ of about 10^{-21} at frequencies below 10^{-1} Hz. In the shorter term, Braginsky and Thorne⁵ propose an interesting detector of intermediate sensitivity (see page 610 of this issue). Their proposed 'skyhook' detector would consist of two masses on each end of a long thin cable with a spring at the centre, the whole system being arranged to orbit the Earth. By sensing the motion of the test masses as transmitted to the spring, a search for gravitational waves could be carried out with an amplitude sensitivity of 3×10^{-17} at frequencies of 10^{-1} – 10^{-2} Hz. This is clearly an experiment to be considered in the near future as it could be ideal for searching for the radiation from a black hole–white dwarf binary system, such as Geminga may be, from which an amplitude signal of approximately 10^{-19} at the Earth at approximately 3×10^{-2} Hz has recently been predicted⁶. Integration of the signal over several months could allow successful detection.

While space experiments are exciting for the future, the potential of ground-based experiments operating over a frequency range from several tens of Hertz to a few kilohertz seems considerable. These experiments are aimed at the detection of gravitational waves from sources such as stellar collapses, coalescing binary stars and fast pulsars. Astrophysical models are somewhat uncertain, but theoretical predictions suggest that a strain sensitivity of 10^{-21} – 10^{-22} over a kilohertz bandwidth should be adequate for the detection of fast-pulsed sources, and 10^{-27} over a narrow bandwidth would be an interesting level for pulsars.

Current experiments use two main approaches. One, the development of the Weber bar detectors using low temperature techniques to reduce thermal noise, is under investigation at a number of places including Stanford University, the University of Louisiana, the University of Western Australia, and at CERN where the University of Rome are mounting a significant effort. The Stanford detector is already achieving a strain sensitivity of approximately 10^{-18} for millisecond pulses with plans to improve this sensitivity by two orders of magnitude over the next five years, and to design a detector system to cover a wide frequency band (a fuller discussion can be found in ref. 7).

The other approach is to increase the signal size by using freely suspended test masses placed a long distance apart. Such an arrangement is inherently wideband. To avoid absolute length measurements, the test masses can be suspended to give two perpendicular baselines in which the gravitational wave signal will induce a differential displacement. The relative length of the two arms can be monitored by laser interferometry, and the optical phase changes can be enhanced by reflect-

ing the light back and forward between a system of mirrors on the test masses, which may form an optical delay line⁸ or resonant Fabry–Perot cavity⁹. A number of prototypes of such detectors are under development. Two of these use optical delay lines (at the Max Planck Institute, Garching, where the arm length is 30 m, and at the Massachusetts Institute of Technology with an arm length of 1 m), and two use Fabry–Perot cavities (University of Glasgow and California Institute of Technology with arm lengths of 10 m and 40 m respectively).

The performance of these prototype detectors is encouraging: strain sensitivity spectral densities of better than 5×10^{-19} Hz^{-1/2} have been achieved by those with longer baselines. And it is generally agreed that they have reached a stage where the construction of detectors with much longer baselines should be considered. The optimum arm length for a detector of this type is a compromise between possible sensitivity, range and flexibility of experiments, and the relative importance of anticipated noise sources. The availability of large areas of flat land and the cost and timescale of building the apparatus also influences the choice. At present it seems that the optimum arm length is a few kilometres. A detector with its masses fitted with mirrors of very low loss, as have been developed for laser gyroscopes, and illuminated by several tens of watts of continuous laser light from an array of high power argon lasers (or a YAG laser), could achieve a sensitivity of close to 10^{-22} for millisecond pulses of gravitational

waves and equally encouraging sensitivity figures for other types of sources.

To set up a useful 'telescope' array on Earth, it would be best to have at least four such detectors. Currently there are plans to have two in the United States developed from the Caltech and MIT prototypes, one in Germany as an extension of the current Max Planck experiments, and one in Scotland as a development of the work at the University of Glasgow. In addition, there is a possibility of a fifth instrument being built in France. If the construction of these detectors proceeds there is a very real chance that gravitational radiation from astrophysical objects will be directly detected within a decade. Towards the end of this time certain of the spaceborne experiments could be giving results in the low frequency part of the spectrum. Evaluation of results from the experiments on Earth and in space can be expected to herald a new and very exciting era for astronomy. □

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Population dynamics

Water fleas as population model

from John H. Lawton

THE most widely used and familiar equation in population dynamics is the logistic¹. It is also the most boring, permitting as it does just one sort of dynamic behaviour — sigmoid growth to a stable equilibrium from low population numbers and monotonic decline to the same equilibrium from above. Many real populations do more interesting things, as Murdoch and McCauley², using *Daphnia* (water flea) population dynamics, demonstrate on page 628 of this issue.

Some of the complexities of real population dynamics can be mimicked by modifying the logistic to incorporate a time-lag in the density-dependent term¹.

$$dN/dt = rN[1 - N(t-T)/K] \quad (1)$$

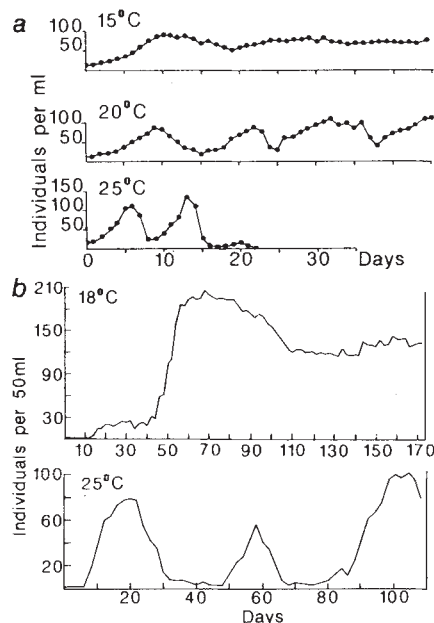
Where N is population density at time t , r is population rate of increase, K is the equilibrium population size, and T is a time-lag in the response of the population to density. When $T=0$ we recover the logistic. With $T > 0$ the modified model generates a range of behaviour¹ including sigmoid growth (for $0 < rT < e^{-1}$) or

damped oscillations ($e^{-1} < rT < \pi/2$) to a stable equilibrium K set by food supply or space; alternatively, stable-limit cycles are generated when $rT > \pi/2$. Density-independent disturbances cause non-cyclic fluctuations round the stable equilibrium, and may sufficiently perturb the damped oscillations to yield 'quasi-cycles'³ — fuzzy, but regular, oscillations.

These more complex behaviours recall those discussed by Murdoch and McCauley². Indeed equation (1) has been invoked to explain the wide range of dynamic behaviour shown by laboratory populations of both *Daphnia* and planktonic rotifers, by postulating changes in the relative values of r and T under different temperature regimes^{1,4} (see figure). To date, analogous ranges of behaviour for field populations have been sought among many biologically disparate taxa and food webs (for example ref. 5). But they have not been demonstrated in the field for one species or closely related set of species under structurally similar conditions —

that is, when essential links in the web of community interactions between prey, predators and competitors remain unchanged. Yet the model explicitly predicts different dynamic behaviour under structurally identical conditions, providing only that key parameter values change in relative magnitude.

Time-lags are inevitable in many population interactions involving real organisms. For example, it takes at least a gestation period for animals to respond to increasing food supplies. But although equation (1) crudely incorporates time-lags, it is still a gross simplification of reality. A step nearer to the truth is a pair of equations — one for the consumer pop-



Laboratory populations of (a) the rotifer *Brachionus calyciflorus* and (b) *Daphnia magna* under different temperature regimes but otherwise identical culture conditions. Increasing temperatures change the dynamic behaviour from approximately sigmoid growth or damped oscillations to regular cycles (a modified from ref. 4; b after D.M. Pratt in ref. 10).

ulation (herbivores) and one for the food (green plants), that is, equations in the form of a Lotka–Volterra ‘predator–prey’ model⁶. Appropriate choice of parameters in such a model again generates the full range of behaviours generated by equation (1) in both prey and predator populations.

Not unreasonably, many field biologists remain sceptical or even hostile^{7,8} to such simple models. They may, after all, appear to do all the right things for entirely the wrong reasons. Therefore, the models need testing. The first step is to find a set of field populations that do some, if not all of the ‘right’ things. As Murdoch and McCauley show, *Daphnia* and their algal prey seem to be ideal subjects to test the pair of equations, providing that obvious pitfalls, such as fish predation, are avoided. What we then seem to have is the two-species field equivalent of a Lotka–Volterra predator–prey model.

Murdoch and McCauley’s *Daphnia*–

algal data challenge the theoretical models in at least three ways. First, and most crudely, do both the relative values of time-lags in the system and *Daphnia* rates of increase agree with the simple predictions of equation (1) for the three classes of populations recognized by Murdoch and McCauley. These are stable (class 1) and cyclic (class 2 and 3) *Daphnia* populations? Second, is it possible to obtain estimates for the parameters of two-species Lotka–Volterra equations, and thus test this more realistic model? For example, is stability (class 1) imposed by mutual interference (intraspecific competition) amongst the *Daphnia*, or by low grazing rates⁶; and do populations in class 2 show quasi- or stable-limit cycles under conditions predicted by the models? Third, as Murdoch and McCauley point out, how is it that class 3 *Daphnia* populations cycle, but not their algal prey? This particular behaviour is not predicted by Lotka–Volterra equations, so either the model or the data are misleading.

It is not difficult to imagine many complexities hidden in these data that may invalidate detailed comparisons with simple models. Examples range from competition between algal species (or between *Daphnia* and other zooplankton), to unsuspected invertebrate predation on *Daphnia* by *Chaoborus* (phantom midges), *Leptodora* (predatory relatives

of *Daphnia*) or water mites, to algal parasites⁹. Indeed, it is extremely unlikely that in the long run, simple Lotka–Volterra models will prove sufficient to model all the complexities of *Daphnia*–algal populations. That is not the function of simple models. Rather, they serve to chart a difficult course between gross simplicity (the logistic) and too much complexity (the field). The models highlight processes that seem fundamental to the dynamics, and signpost the next round of field observations and experiments. In short, *Daphnia* and algae present ecologists with an opportunity to test some important, general, widely used but often maligned models, but at the same time keeping both feet firmly planted in the muddy complexities of the nearest pond. □

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Glaciology

Contrasts in Vostok core — changes in climate or ice volume?

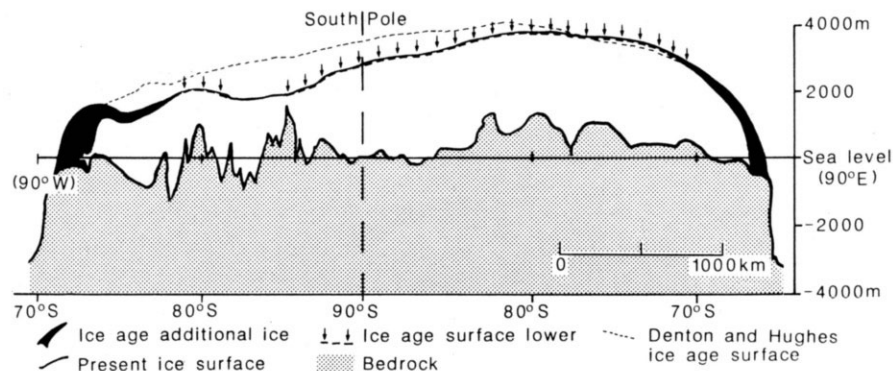
from G. de Q. Robin

CORES from the major ice sheets provide fossil samples of the Earth’s atmosphere and yield information on past climates and the mechanisms of glaciation. But obtaining such cores has never been easy, and a really long record has proved elusive. Now Soviet engineers have drilled an ice core of high quality to 2,083-m depth at Vostok station at 78°S, the coldest inhabited place on Earth. This core from East Antarctica is the first to span the last interglacial and recover ice from the preceding glacial period. Papers on p. 591 and 616 of this issue provide an exciting glimpse of results to come from geochemical studies of this 150,000-year climatic record^{1,2}.

Dating of the ice core is based on my observation that current accumulation rates in central Antarctica vary with mean atmospheric temperature above the ground inversion³. These temperatures are reflected in the $\delta^{18}\text{O}$ profile determined by Lorius *et al.*¹ and are used by these authors to extrapolate from present to past accumulation rates. The agreement of the resultant date for the last interglacial with that obtained by other methods suggests the method is satisfactory, as

does the agreement with accumulation rates deduced from the ^{10}Be studies of Yiou *et al.*² Some corrections to allow for increased atmospheric transport of dust⁴ and thus of water vapour during the last glacial maximum may be desirable. The new method is an improvement on dating of marine sediment cores, where uniform accumulation rates between horizons marking prominent climatic boundaries 20–60 kyr apart are usually assumed.

Since the annual accumulation around Vostok is less than the surface roughness due to sastrugi (irregularities caused by the scouring action of windborne ice particles), deposition noise levels⁵ in the $\delta^{18}\text{O}$ record are high. The sampling length of 1.5–2 m (equivalent to 100–200 years) reduces this noise to around $\pm 0.5^\circ$ per thousand (standard deviation). This is similar to deposition noise levels found by comparing measurements from 20 shallow bore holes 600 km away at Dome C station⁶. Separate measurements at 25-m intervals along the whole core (Fig. 1 in Ref. 1) lie mostly within two standard deviations of the continuous curve below 1,406 m, although a difference of four



Suggested zones where the Antarctic ice sheet was thicker or thinner (that is, the ice surface was higher or lower) during the last glacial maximum than at present⁸. This is compared with corresponding surface elevation estimates from Denton & Hughes¹⁶. Thicker ice in coastal regions is due to increased grounding of ice as sea level falls during an ice age. Lower ice surfaces inland show responses to lower accumulation rates suggesting that surface levels of ice streams and glaciers discharging ice from central regions undergo little change or even fall slightly¹⁰.

standard deviations occurs at the interglacial maximum. Within these limits, most fluctuations in the $\delta^{18}\text{O}$ profile can be related to global climatic factors⁷. A notable exception is isotope Stage F; the Vostok record shows much colder conditions than do other marine, ice and terrestrial data for that time.

A rough periodicity of around 40 kyr in the $\delta^{18}\text{O}$ profile, in phase with summer insolation changes at 80°S, strongly suggests a connection with the 'Milankovitch' orbital variations. Together with the similarity in isotope composition at the four minima recorded, this led Lorius *et al.*¹ to conclude that the $\delta^{18}\text{O}$ profile at Vostok primarily records climatic (regional temperature) changes and that effects of changes in surface elevation are small. This relative emphasis on a regional rather than a global explanation of the Vostok climatic record is due in part to the difficulty of finding a satisfactory mechanism of heat transfer between the hemispheres. However, probable changes in the latitude of the effective meteorological equator, in response to changes of radiation balance over the Earth's surface during ice ages, provide an equivalent mechanism (Radok, U. & Riehl, H. personal communication). A global response therefore remains plausible.

The alternative possibility, that ice surface levels over central Antarctica were lower (perhaps by 200–300 m), is supported by recent studies⁸ of factors affecting mean sea level — which may have been 5–7 m higher during the last interglacial. Applying the statement that "climate during the last interglacial was extremely similar to the present" (ref. 9) to the Vostok isotope profile leads to a similar conclusion. The difference between present day and interglacial $\delta^{18}\text{O}$ values could then be interpreted as indicating that the surface elevation around Vostok was about 300–350 m lower during the interglacial than now. If extrapolated to most of central Antarctica, the reduced volume of water bound up in the Antarctic ice sheet would be sufficient to explain the higher sea level, even though the climate

and glacier volumes other than in central Antarctica were the same. This hypothesis avoids the difficulty either of having to melt large sections of the Greenland and Antarctica ice sheets or of having the West Antarctic ice sheet collapse with little change of climate, in order to provide the necessary water. The higher sea level at this time also need not be interpreted as an indication of higher temperature⁹.

Evidence from several sources⁸, including Lorius *et al.*⁴ and Drewry¹⁰, suggests that surface levels in central Antarctica were somewhat lower during the last glacial period than at present (see figure). Because the accumulation rate is very low, the response time of ice thickness to climatic change in central Antarctica is probably tens of millennia compared to a few millennia in coastal regions. Consequently the ice thickness at the start of an interglacial will reflect conditions during the previous glacial period. Marine cores and terrestrial evidence indicate the glaciation from around 200–135 kyr was both colder^{11,12} and the colder temperatures were more prolonged¹¹ than that preceding the present interglacial. Hence average surface elevations in central Antarctica could have been 200–300 m lower around 135 kyr BP than during the Holocene. Applying a similar argument to isotope Stage F suggests that ice then may have been up to 200 m higher than at present following an interglacial buildup. Regional climate would then be some two degrees warmer than that deduced from the assumption of constant elevation in central Antarctica. This would reconcile the Vostok $\delta^{18}\text{O}$ values with general climatic records.

A problem with the surface elevation changes suggested above is that they are larger than expected from steady state glaciological theory. This could be explained by combining aerial and temporal variations of ice accumulation rate in Antarctica. Data from Lorius *et al.*¹ suggest an approximate ratio of 100:60 between interglacial and glacial accumulation rates in central Antarctica. This is supported by the findings of Yiou *et al.*² on precipitation rates. With present day accumulation

rates of 2.5 cm of ice per year at 3,500-m elevation around Vostok and around 10 cm of ice per year at about 2,500 m, the corresponding rates during a glacial period would be 1.5 and 6 cm per year respectively. During an interglacial period of say 20,000 years, 200 m more ice would accumulate at Vostok than during an equal time in a glacial period. At 2,500-m elevation the difference could be as much as 800 m. This would reduce an initial 1,000-m elevation difference between two sites to 400 m after 20,000 years of interglacial conditions, if ice flow were unchanged. Since flow is driven by a shear stress proportional to the product of ice thickness and slope, the dominant influence will not be the increased ice thickness but the decreased surface slope. This will decrease flow once an interglacial period starts and will further increase ice thickness. Such feedback could help explain the rather large elevation changes that it is possible to deduce from the isotope date. It could also explain the present relatively low surface slopes on central Antarctica, which have been cited as evidence of a former surge of the ice sheet¹³. The small positive mass balance in the Antarctic ice budget, noted recently¹⁴, would also be consistent with central elevations that are increasing slowly.

The question of whether the results from Vostok should be explained mainly in terms of climatic effects on an ice sheet of constant surface elevation or by ice volume changes in central Antarctica may be resolved¹⁵ when more detailed isotope and total gas volume measurements become available. Whatever the answer, the Vostok core will have been of considerable importance in unravelling the history of the past global climate, ice volume and ocean levels over a full glacial cycle. □

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Gene control

False starts in translational control of gene expression

from Tim Hunt

THE control of eukaryotic gene expression at the stage of translation of messenger RNA into protein has always been a theoretical possibility, but surprisingly few clear examples have been identified. And even in the well-studied cases, detailed molecular explanations are almost wholly lacking. However, a number of cases of selective translation of mRNA are known and interesting clues to the mechanisms that underlie this kind of regulation are beginning to emerge from recent studies.

These studies focus attention on a number of unanswered questions about how the translational machinery operates. For example, it is known that translation is initiated at an AUG codon in the mRNA; but if there is more than one AUG, how does the ribosome know which to use? What determines the frequency with which a particular message is read? When a ribosome has finished translating an open reading frame in a message, can it start up again if a suitable AUG follows in that same mRNA? Can cells keep a mRNA altogether untranslated in some circumstances, but translate it if the circumstances change? All these questions are yielding interesting new answers, thanks mainly to nucleotide sequence analysis of natural mRNAs and the ability to test hypotheses by making alterations to these mRNAs or by constructing synthetic messages using recombinant DNA methods.

Scanning

According to the 'scanning' hypothesis formulated by Marilyn Kozak¹, the eukaryotic ribosome moves down the mRNA from the 5' or upstream end (which is usually capped) until it encounters the first AUG codon, whereupon translation is initiated. In more detail, 40S ribosomal subunits bearing Met-tRNA_i move along the mRNA until the anticodon of the Met-tRNA_i engages with the first AUG, whereupon the 60S ribosomal subunit joins on, the next tRNA is inserted and the first peptide bond forms. Then the (now) 80S ribosome moves down the mRNA, translating it into protein until a termination codon is reached. This causes the protein to be released from the ribosome, and the ribosome to leave the mRNA, presumably by reversing the initiation process and dissociating into its subunits. These processes, which are not very well understood, may in the light of recent findings, prove to be important in the translational control of protein synthesis: for it turns out that the coding

regions of certain mRNAs are preceded by up to four starts and stops.

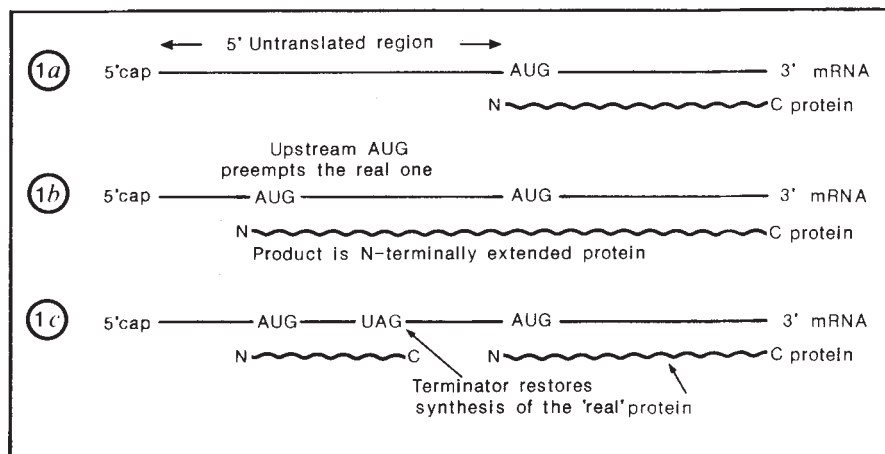
As the Kozak model predicts, the first AUG is used in over 90 per cent of mRNAs whose sequences are known. It usually occurs between 50 and 100 residues from the 5' end of the message, often as the consensus sequence PuN-NAUGG. However, in about 5 per cent of cases, one or more AUGs occur upstream of that which leads to synthesis of the supposed product of the message. What happens when ribosomes encounter these false starts? And, if they are read, what are the consequences?

These questions have been answered in part by deliberately inserting AUGs upstream of the 'real' AUG in mRNAs². In general, it seems that an inserted AUG preempts the natural one, in accord with the scanning hypothesis. If the reading frame of the false start is the same as that

within the first 200 residues of a 2,000-nucleotide mRNA, two AUGs give rise to different proteins formed by alternative starts in the same reading frame, while a third protein of completely different length and sequence derives from an AUG in a different reading frame³. A rather similar situation occurs in adenovirus, where an mRNA from the *E1b* gene specifies alternative proteins according to which AUG is used⁴; the first occurs very close to the 5' end of the message and is often ignored in living cells, but it seems to be preferred in cell-free translation systems. These cases do not strictly qualify as examples of translational control, because there is no evidence that the frequency of initiation from the alternative AUGs can be regulated. Rather, they seem to represent cases of economical use of the viral genome, made possible by exploiting wrinkles in Kozak's rules.

Translational control

By contrast, it is now known that expression of the yeast *GCN4* gene (involved in the General Control of Nitrogen metabolism) is regulated primarily at the level of translation^{5,6}. The *GCN4*-encoded protein is a positive transcriptional regulator of many genes involved in amino-acid



of the real one, the result is an amino-terminally extended protein (compare Fig. 1a with 1b). But if, irrespective of the frame of the upstream AUG, termination signals (the codons UAG, UAA or UGA) occur between the false and real starts, ribosomes resume reading the real message, suggesting that they remain attached to the mRNA after terminating synthesis of the first protein, and are able to start up again if a suitable AUG presents itself within a reasonable (but undetermined) distance (see Fig. 1c).

While these experiments support Kozak's rule, there are several cases of different natural AUGs in the same mRNA being read to give alternative products. This suggests that ribosomes can miss an AUG, perhaps if it is embedded in a sequence that is poorly matched to Kozak's consensus sequence.

The mRNA coding for the P and C proteins of Sendai virus is a good example;

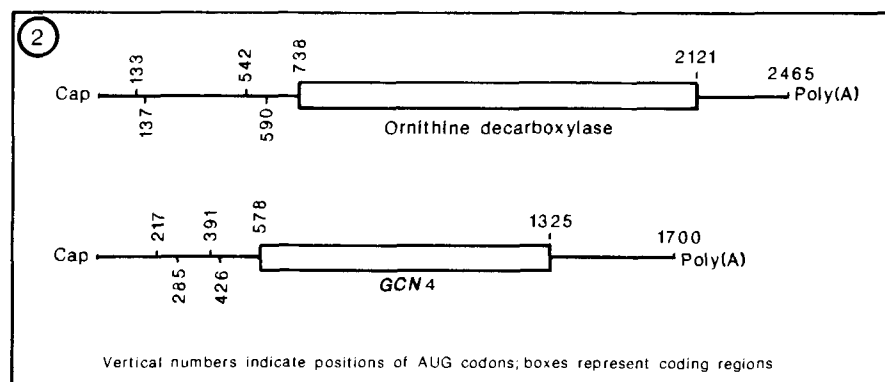
Its synthesis is boosted something like 100-fold when yeast is starved of any essential amino acid. Its level of expression depends on the activity of a repressor specified by the *GCD1* locus, whose activity is in turn somehow regulated by the *GCN1*, 2 and 3 loci. Until recently it was assumed that this complex regulatory hierarchy operated at the stage of transcription, but it now emerges that the level of *GCN4* mRNA stays essentially constant regardless of the nutritional status of the cell: what changes is the rate at which it is translated. Clues to the mechanism are evident in the structure of the mRNA. It has a 5'-untranslated sequence which, at 577 nucleotides, is much larger than normal, and there are four AUG codons upstream of the 'real' one, each followed by a termination codon two or three codons later. A deletion of 280 nucleotides that removes all the upstream AUGs leads to greatly increased translation of the message, consis-

tent with the idea that the upstream AUGs limit translation in some way. Experiments in which point mutations are made in the AUG codons are under way; until the results of these emerge, it will not be clear whether the upstream AUGs are actually read by ribosomes. But, assuming this is the mechanism, how is it triggered by amino-acid starvation?

Amino acid starvation is almost certainly sensed by ribosomes themselves. In bacteria, ribosomes that get stalled on mRNA waiting for a missing aminoacyl-tRNA convert a very significant fraction of intracellular GTP into pppGp and pppGpp. These compounds reach high levels and activate the 'stringent response'. No analogous conversion of low molecular weight phosphorylated compounds has ever been detected in eukaryotic cells despite many searches, so something different must be going on in yeast. The other bacterial paradigm, 'attenuation', acts at the level of transcription and is clearly not directly applicable. Besides, there does not seem to be any correspondence between the amino acids specified by the tiny reading frames following the upstream AUGs and those whose depletion turns on the *GCN4* gene product synthesis. Thus we must await further elucidation of the molecular modes of action of the *GCD1* and the other *GCN* gene products before understanding much more. Probably they are closely involved in the protein synthesis machinery, a hypothesis strengthened by the inviability of *GCD1* mutants.

A strong echo of this story returns in the report of the surprising sequence of a full-length cDNA clone corresponding to ornithine decarboxylase⁷, an enzyme that is involved in polyamine biosynthesis and is extremely rapidly produced by cells that have been stimulated by hormones and growth factors. The interesting thing about its mRNA is that it contains a 737-nucleotide 5'-untranslated region containing four AUG codons upstream of the long open reading frame, followed by terminators 16, 2, 4, and 10 codons later. In broad outline this is highly reminiscent of *GCN4* mRNA (see Fig. 2). The mRNA for ornithine decarboxylase is not very efficiently translated *in vitro*, suggesting that ribosomes rarely reach the proper coding region, but it is not known whether its translational efficiency changes with the physiological state of the cell; if so, does the long leader plays a part in the regulation? It would be very interesting to put this mRNA into yeast and see if amino-acid starvation made any difference to its expression.

There are still relatively few cases in which specific translational control has been shown to occur in eukaryotes, which is one reason the *GCN4* example is so exciting. Several well-known examples, such as the displacement of normal cellular mRNA and the induction of new protein synthesis after heat-shock, or virus-



induced shut-off of host protein synthesis, remain poorly understood at the molecular level. There are several examples of 'masked' mRNA in eggs and oocytes — that is, messages that are very abundant but essentially untranslated until after fertilization. The mRNA that encodes the small subunit of ribonucleotide reductase in clam and sea urchin eggs is a very good example⁸. We do not have the complete sequence of any of the mRNAs that show this behaviour, nor any other possible clues as to the mechanism involved. Another rather striking example of developmentally timed 'unmasking' of mRNA is provided by *Xenopus* fibronectin mRNA, present in unfertilized eggs but untranslated until about the time of the midblastula transition, 6–7 hours after fertilization⁹. It would be well worth looking at the 5'-untranslated leader of this message to see if it gives any clues to the masking mechanism. A recent and particularly spectacular example of translational control is that in *Volvox*, where the pattern of protein synthesis changes dramatically in response to light⁹.

Pointers

Two other helpful points about 5'-untranslated regions have been discussed in recent publications. First, when RNA sequences capable of forming strong secondary structure (inverted repeats rich in GC base pairs) are engineered into the 5'-untranslated sequence of a mRNA, translation of the mRNA is reduced¹¹. Second, annealing the untranslated sequence (including the initiation codon) with complementary DNA or RNA completely stops ribosomes finding the start of the mRNA, though once ribosomes start polymerizing amino acids, they are apparently able to unwind or peel off such double-stranded impediments to their progress^{12,13}. Control mechanisms could exploit these features; the 5'-untranslated region of mRNA is the first place to look for possible regulatory sequences.

But neither argument nor experiment has yet settled the role of long 5'-untranslated sequences, with or without upstream AUGs or secondary structure, in the regulation of translation of mRNA. It is certainly not the case that all mRNAs with long leaders are very inefficient; encephalomyocarditis viral RNA which has

an exceptionally long untranslated segment, which includes several upstream AUGs, is one of the most active messages known, both in reticulocyte lysates and infected cells. By way of contrast, the case of *c-myc* mRNA, which normally has a 5'-untranslated region of well over 400 nucleotides, seems to be controversial. It has been suggested that chromosomal rearrangements that cause shortening of this region result in increased expression of the protein by increasing the frequency of translation of the mRNA¹⁴, but two very similar experiments designed to test this directly (using synthetic mRNAs synthesized *in vitro* from cloned templates by SP6 RNA polymerase) have given conflicting results^{15,16}. An analysis of intact cells, which is anyway much more difficult, has not resolved the conflict¹⁷.

Thus, while it remains easy to imagine the existence of proteins that act as specific translational repressors and enhancers by binding to particular regions of the primary or secondary structure in the 5'-untranslated regions of mRNA, not one authentic example has yet emerged from years of studying mRNA and messenger ribonucleoprotein particles (excluding the poly(A)- and cap-binding proteins, which do not seem to play regulatory roles). I hope yeast will provide the real thing. □

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Photosynthetically active radiation — a new unit

SIR—It is meaningful and useful, and now fashionable in studies involving photosynthesis, to express light intensities as micro-einsteins of photosynthetically active radiation per square metre per second: $\mu\text{E m}^{-2}\text{s}^{-1}$ (PAR). This unit is not a standard international one, as is a mole of photons. It could be confusing since to physicists the Einstein number is a velocity relative to that of light. In fact, it is obviously not a purely physical unit but a physico-botanical one, since spectra of photosynthetically active light differ among green plants, red and brown algae, diatoms, cyanophytes and other photosynthetic bacteria according to their respective complements of photosynthetic pigments: chlorophylls, bilin pigments, and so on. It is awkward to type, since it involves negative exponents and a Greek letter found on few typewriters. It is also a long phrase to use in common parlance, and referring to it briefly and colloquially as a "micro-einstein" is wrong.

I therefore suggest that for physico-botanical usage, in such fields as plant physiology, limnology and oceanography, we use a new nominal unit, the albert: 1alb = $1 \mu\text{E m}^{-2}\text{s}^{-1}$ (PAR). (This continues to commemorate Albert Einstein.) It is a derived unit, like a joule or a roentgen. Very approximately, one kilo-lux is equivalent to about 20–40 alb, while full sunlight provides a light intensity in the range of 2,000–4,000 alb (depending on the latitude and on the kind of photosynthetic system involved).

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Splicing and the evolution of introns

SIR—The recent suggestion by Cavalier-Smith¹ that introns have evolved by duplicative transposition within eukaryotic genomes is one which we believe deserves serious consideration. We would, however, like to offer an alternative to the model of transposition at the level of DNA. We propose that if introns are indeed the vestiges of transposable elements, they represent a class of retrovirus-like transposons or retrotransposons². The attraction of this latter model is that it gives the splicing mechanism a central role in the evolution of introns. There is a growing body of evidence to suggest that splicing is intron encoded^{3–6}. Consequently, it is reasonable to look for evolutionary explanations of the splicing mechanism in the context of the evolution of the introns themselves.

In our view, the ancestral intron genomes were replicated into RNA copies simply because of their insertion within

transcriptionally active regions of the host genome. Splicing was necessary not only to minimize negative effects on host gene expression^{1,7} but also, perhaps more importantly, to generate new copies of the intron genome free of flanking exon sequences⁷.

These spliced intron copies were then available for reverse transcription and reinsertion elsewhere in the genome. Most modern introns have probably lost much of their original genetic content and may be considered as degenerate evolutionary relics. An exception is the set of splicing signals which must be retained because of its importance to host cell survival.

Finally, we would like to point out that any particular model of the origin of introns does not argue for or against a subsequent short-term or long-term evolutionary role for these elements within the eukaryotic genome.

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Current density and permittivity

SIR—John Maddox (*Nature* **316**, 101; 1985), in his article on Maxwell's displacement current should have been more careful with his electrical units. J is not a current but a current density measured in A m^{-2} . The preferred name for ϵ is permittivity; it is not a scalar but in general a second rank tensor. The name dielectric constant for ϵ should be avoided, as ϵ usually varies with temperature and frequency and can vary with applied field strength. (See *Quantities, Units and Symbols*, Royal Society, London, 1975 and addenda 1981).

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Null hypotheses and the misuse of statistics

SIR—Van Valen pleads for greater freedom for the applied scientist constrained by the "narrow Neyman–Pearson view of statistics as hypothesis-testing" (*Nature* **314**, 230; 1985). He advocates a more flexible use of statistics and although in the main we do not disagree with this, our own experience in medical statistics leads us to believe the problem is more fundamental than misuse of null hypotheses.

Statistics involve three ingredients — technical statistics, language and method. Mastery of all three would be ideal. Mastery of technical aspects is a minimum

requirement, based on the organization of ideas according to established recipes defined by previously encountered 'typical' research problems. The technical element then is more adapted to provoking action than to reflection, and if we agree that reflection followed by action underlies scientific progress, then we might reasonably consider it perverse for a researcher to be incapable of formulating hypotheses in the absence of stimuli from a computer.

The language of statistics is that of mathematical models and probability theory underlying the calculations. With the advent of computer packages and a much wider access to P -values, increasingly solicited by journals, mastery of the language is rapidly becoming seen as a specialist area only of use in unravelling the most complex situations.

Method is concerned with the fundamental approach and not immediate operational questions. Under this heading we have for example Bayesian techniques, parameter estimation and . . . hypothesis testing. The mistake is to regard these approaches as fundamental principles and to try and squeeze all problems under one or another heading. Worse still is to try and answer questions using one approach which can only be answered using another. To take epidemiology as an example, we almost have to admire the ingenuity whereby, to avoid modelling, problems are formulated in terms of the most untenable null hypotheses. Researchers aware of differential effects and yet with hopelessly inappropriate means for quantifying them find themselves writing $P < 0.0001$ to underline an effect of possibly greater importance than one for which, for instance, $P < 0.01$. Such an expression can find little motivation or meaning in the asymptotic framework of the distribution theory underlying any test, even when appropriate, and yet the optimistic expression $P < 10^{-7}$ is by no means unknown in the medical literature.

To would-be users of statistics it should be made clear that the acceptance or rejection of a null hypothesis does not establish scientific knowledge with the same degree of conviction and this is entirely consistent with Popper's outlook. Unfortunately we are tempted to overlook this fundamental logical asymmetry: thus while some will invoke a non-significant result as evidence for the equivalence of treatments others will invoke a non-significant result as proof of the comparability of patient groups before treatment. In contrast, a significant result will almost always be given a weight disproportionate to its scientific import, the constant of disproportionality varying inversely with the level of significance.

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Between fact and passion

Alan Cottrell

No Immediate Danger: Prognosis for a Radioactive Earth. By Rosalie Bertell.

The Women's Press, 124 Shoreditch High Street, London E1 6JE: 1985.

Pp.435. Hbk £11.95; pbk £5.95.

Power Production: What are the Risks? By J.H. Fremlin.

Alan Hilger: 1985. Pp.318. £22.50, \$33.

How extraordinary it is that two such books as these, written by obviously sincere, intelligent, dedicated and independent authors on the same scientific subject — the risks to the general public of nuclear radiation — should reach such diametrically opposite conclusions. The ground they cover, particularly the biomedical effects of radiation from nuclear power production, is virtually the same, although Dr Bertell's text extends to nuclear weapons production and testing, which she regards as inseparable from nuclear power, and Professor Fremlin's covers in some detail the risks from other methods of energy production, which are dealt with only cursorily and optimistically by Dr Bertell. The difference between the books is all the more extraordinary considering that, for both authors, the preservation of the natural world and its resources is a major objective, as is also the raising of the standard of living in the Third World. Furthermore, both see nuclear warfare as a total catastrophe for everyone. Yet, for Fremlin nuclear power is one of the few bright hopes for mankind on an overcrowded planet, whereas for Bertell it is an ultimate evil, to be attacked with the zeal of a crusader.

Fremlin's is a scientist's book. Cool analysis and numerical estimation are its weapons, used with great skill and elegance to expose many a foolish notion or false argument; for example, that 1 kg of plutonium would be sufficient to kill off all life on Earth. Thus

The natural uranium, radium and polonium in the ocean give off about 40 alpha particles per gram of sea-water per year. One kilogram of plutonium distributed in the oceans of the world . . . would give about 1 alpha particle per gram in 50,000 years [p.263].

In addition to his opposition to the unnecessary use of fossil fuels, Fremlin's main bias is "in favour of facts" and it is this that has led him to expose and attack false arguments used by anti-nuclear campaigners and disgracefully irresponsible treatments by the media, such as the blatant distortion presented by Yorkshire Television in Britain on 1 November 1983 (p. 275).

Dr Bertell is a scientist but hers is not a scientific book. It is a tirade and manifesto, brilliantly written, but with the set mind of the zealot. Passionate description and harrowing anecdote are her weapons, used with great skill and vividness to re-

mind the reader of what must never be forgotten; of the immense horrors of Hiroshima and Nagasaki. But Bertell treats inconvenient facts more disdainfully than Fremlin. Thus, about the genetic effects of radiation she writes

the data accumulated at Hiroshima and Nagasaki did not give the desired answers. Either through ineptitude or loss of survivors of the bombing . . . the researchers failed to find any severe genetic ills . . . [p.47].

If the facts do not suit the argument, so much the worse for them! The passion also

IMAGE
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FOR
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REASONS

Power of good? Part of the British Nuclear Fuels Sellafield site in Cumbria, UK.

leads to some surprising assertions. Thus, mankind's nuclear activities have caused acid rain (p.56), the change in the El Niño ocean current (p. 102), and earthquakes (p. 163), as well as 16 million casualties. It is all a conspiracy of militaristic governments, especially Western ones. Even Pearl Harbor was a tactic of the United States designed to strengthen the fighting attitude of American people (p.151).

In a sense, these two books re-enact the classical contest between heart and head in their attempts to find a better future for mankind. Bertell advocates the Utopian future of a global village in which

the fundamental human needs for food, shelter, clothing, medical care, education, work and social dignity are met, so that the tremendous human potential for creativity, art,

science and culture is freed [p.295].

Magnificent, but is it not what mankind has always wanted? And how do we get it and maintain it? Bertell's answer is that

the money for food and celebration of life is being needlessly squandered on weapons and unneeded technological mega-projects [p.296].

Beating swords into ploughshares has been a desirable goal for endless generations and in each new one it has stumbled against the same old problem: human aggressiveness. This is why the heart is not enough. Its solutions are idealistic, planned for angels, not human beings. The head has also to play its part, to seek realism and practicality in the face of human nature as it stands. Peace with goodwill is what Bertell wants, but these may be beyond mankind's reach at present, in which case the grudging peace without goodwill that now exists between the nuclear superpowers may be the best that we can realistically have. But for Bertell, the concept of deterrence is simply not admitted; she is sure that the nuclear powers are preparing to go to war, not trying to dissuade each other from war, in spite of the fact that the European corner of the globe—traditionally a most belligerent region—has enjoyed an unprecedented period of freedom from war throughout the nuclear age.

Fremlin's goals are more limited but more attainable. The people of the world need energy. The big and rapidly multiplying poor in country villages in Africa and Asia need cooking fuel, clean water, practical education and time.

Kerosene and coal are alternatives to the destruction of the forests and the rich countries should be systematically planning to conserve these as effectively as possible and as soon as possible. Compared with the disastrous effects inevitable in an overcrowded world, none of the health hazards of any form of energy production are of any importance at all . . .

The Third World can be helped in this respect

. . . if the richer countries use as much wind and solar energy as they can, develop nuclear energy . . . and use nuclear electricity to replace oil, coal and gas . . . [p.280].

Both books brilliantly expound their author's views but it seems likely that both will fail to persuade unsympathetic readers to change theirs. Fremlin's sophisticated rapiers, needle-sharp though they are, are unlikely to pierce the hermetic seals in which the anti-nuclear protesters have insulated themselves against contamination from uncongenial scientific facts. And the unrelieved damning of all things nuclear by Bertell appears too obsessive to be convincing. To this reader at least, the truth seems less likely to dwell among her passionate advocacies than among Fremlin's cool arithmetic. □

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Myth, magic and the common solution

Anthony W. Clare

Sherlock Holmes and the Case of Dr Freud. By Michael Shepherd. Tavistock: 1985. Pp.31. Pbk £2.95, \$5.95.

IN Nicholas Meyer's *The Seven Per Cent Solution*, Sherlock Holmes, suffering from a severe mental disorder with delusional ideas concerning the machinations of a harmless Professor Moriarty, meets and is treated by Sigmund Freud who is at the time a young physician in Vienna earning a reputation for himself treating the neuroses by an exciting new method of exploration. Cured by the treatment, Holmes joins forces with Freud and together they engage in a successful hunt for a villain who is attempting to precipitate a world war.

Michael Shepherd, Professor of Epidemiological Psychiatry at the Institute of Psychiatry in London, opens what is a deceptively temperate essay with this revisionist view of Conan Doyle's hero. The reason he does so is quite simply that he is about similar business with regard to the sage of Vienna and the founder of psychoanalysis. Sherlock Holmes made the external world coherent by solving mysteries using a method of procedure

which, although appealingly labelled as deductive and logical, was in actual fact intuitive and illogical. However, so attractively human was it that it was and continues to be enormously popular and enjoyable in contrast to the numbing tedium of the real process of detection. Freud, likewise, appeared to make the world comprehensible by solving internal mysteries by a method which, Shepherd argues, was no less intuitive and illogical whatever the claims for scientific status made by Freud and his successors on its behalf. In precisely the sense that Holmes is a caricature of the real life detective, so Freud is a caricature of the psychiatrist.

However, there is more to the link between the fictional private eye from Baker Street and the actual therapeutic ear from the Berggasse. Both Holmes and Freud are the stuff of which heroes are made. Shepherd quotes Brigid Brophy and her view that the cause in which the modern detective employs his method and skill remains the same as that in which the Greek hero used his magic powers — "the deliverance of the population from a threat". In sociological terms, the detective story offers its readers a reassuringly comprehensible and predictable world in which those who try to upset the established order are always detected and punished. Freud's great case-histories read like stories in a Holmesian omnibus and a contemporary novelist, D. M. Thomas, has gone so far as to argue that in his clinical accounts Freud was often fictionalizing, as intent on getting "a good story, a well-shaped classical Greek story, as to get at the truth". Freud and Holmes, Shepherd suggests with more than a touch of mischief, are examples of Nietzsche's *ubermensch*, the new man, detached, quasi-omniscient, a contemporary hero of an ancient legend.

It is an effervescent, provocative, humorous yet profoundly serious argument, and Shepherd elegantly draws on the sympathetic effusions of many of the literary supporters of psychoanalysis to illuminate his view that psychoanalysis, wherever it ultimately belongs, appears more at home within the sphere of myth than of medicine. Others have had the same idea (for example an equivalent argument is mounted in Ernest Gellner's recently published *The Psychoanalytic Movement*), but few have made the case with such an economy of words and breadth of scholarship. To those who know Shepherd and his work, this lively appetizer will maddeningly remind them of the more substantial meal he could cook if the spirit but moved him. Disciples of Freud and psychoanalysis may well breathe a sigh of relief that to date it has failed to do so. □

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The fitful gleam

P. W. Hawkes

Statistical Optics. By Joseph W. Goodman. Wiley: 1985. Pp.550. £49.85, \$59.80.

FLICKERING, glistening, twinkling, shimmering: the literary vocabulary of optics should lead us to expect statistics to play a large part in the subject even if the scientific language is more sober. There are indeed branches of optics — coherence, speckle, some image restoration methods, image compression, some aspects of pattern recognition, weak signal detection — which require a thorough knowledge of statistics, but the first books on probability with special reference to optics have appeared only very recently. The first, *Probability, Statistical Optics, and Data Testing*, written by B. R. Frieden and published by Springer-Verlag, appeared in 1983 and set a very high standard. Although Goodman's new text contains some material that is also to be found in Frieden, the two books are otherwise very different in coverage.

Statistical Optics opens with a brisk account of probability theory and random processes, written in the spirit of a revision aid. Individual chapters are then devoted to first-order statistics, the traditional study of temporal and spatial coherence in terms of second-order statistics, situations in which higher-order coherence arises, image formation with partially coherent light and the phase problem, propagation through random media and, finally, the detection of weak optical signals.

The ratio of optics to statistics is uneven. The chapter on first-order properties is a successful attempt to inculcate statistical thinking habits into students of optics, through the study of thermal light and laser light. The following chapter, on coherence, follows the reasoning made familiar by such standard texts as Born and Wolf's *Principles of Optics* (Pergamon, 1980) and the same is true of most of the later chapter on effects due to partial coherence in imaging systems. The latter concludes with a very brief account of phase retrieval and a slightly longer section on speckle. In the case of phase retrieval, there is hardly any discussion of the extremely important problem of noise in the various reconstruction methods, which is a disappointing and surprising omission because this is an eminently statistical topic. The chapter on higher-order coherence (of which subject it is distinctly less easy to find an elementary account) is much more useful.

The two concluding chapters, on imaging through randomly inhomogeneous media and fundamental limits in photoelectric light detection, are genuine statistical optics, in which the interdependence between the two fields is shown clearly. Goodman leads us through the theory of

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random amplitude and phase screens and then goes on to examine propagation through thick layers of turbulent material, typically the atmosphere. The discussion concludes with an analysis of stellar speckle interferometry. In the final chapter, he presents the semi-classical theory of photodetection, including photo-counting statistics, the notion of degeneracy parameter, and noise limitations in various kinds of interferometry.

The text is clearly and interestingly written, but one serious criticism remains (probably reflecting more on the status of optics in university physics curricula than on Professor Goodman): this is not a complete optics course, so students must presumably attend others in which they could reasonably be expected to learn about partial coherence and its propagation. Such topics could therefore have been covered much more concisely, leaving space to deal thoroughly with material more likely to be new: the phase problem, super-resolution, and the host of statisti-

cal methods and notions used in image processing.

For physicists, then, *Statistical Optics* will be welcome as a guide to the parts of statistics needed in optics, but it does not go deep enough into the various applications for the student who plans to make optics his career, or at least the subject of his PhD research. For the electrical engineering community, however (and Professor Goodman's chair is in this subject), the book probably provides a useful initiation into the optical world for those whose preoccupations bring them into contact with the topics covered but who do not need to go into them too deeply. There is thus still room for a book in which the author assumes that his readers have mastered the statistics in this book, or in Frieden's, and are now ready for a thorough examination of statistical optics. □

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A life in theory

Esther R. Phillips

The Mathematics of Sonya Kovalevskaya. By Roger Cooke. *Springer-Verlag*:1985. Pp.234. DM96, \$29.80.

SONYA Vasilievna Kovalevskaya (1850–1891) was a mathematician, poet, novelist, dramatist, political tract writer and, some would add, revolutionary. She embraced the nihilism of the 1860s, with its optimistic, perhaps naïve, faith in the power of science and reason to establish a better society, and set out, against great odds, to obtain the university education that would enable her to take part in the building of a new society.

In the course of her turbulent and sometimes melodramatic life, Kovalevskaya contracted a fictitious marriage (1868) which permitted her, along with her sister and friends, to travel abroad to receive the university education denied them in Russia. Barred from attending classes at the University of Berlin (as were all women), she persuaded Karl Weierstrass, one of the leading mathematicians of his time, to give her private lessons covering the material he presented in his university lectures. Her studies were abruptly interrupted in 1871 when she journeyed to be at the side of her sister, an active participant in the short-lived Paris Commune; here Kovalevskaya served as a nurse for five weeks.

Returning to Berlin she resumed her studies, and under Weierstrass's direction she received a doctorate in mathematics (the first awarded to a woman) in 1875. A five-year break from mathematics followed, during which she returned to Russia, had a child, engaged in disastrous real

estate speculations, wrote several non-mathematical works and plunged into a frenetic social life in Petersburg. With the help of Weierstrass and G. Mittag-Leffler (also a former student of Weierstrass) she obtained a position (the first woman to do so) at the University of Stockholm, where, in 1889, she was appointed professor for life. The previous year she had received the Bordin Prize of the Paris Academy of Sciences for her memoir on the rotation of rigid bodies.

Finally, in 1889 she was elected to the Petersburg Academy of Sciences (again, a first for a woman) as corresponding member. At the time of her sudden and untimely death in 1891, Kovalevskaya was at the height of her career — an “international celebrity”, as Roger Cooke describes her (p.116).

In the book Cooke has provided a complete account of Kovalevskaya's work and a sympathetic, but not adulatory, assessment of it. Although numerous articles with biographical details have been published, there is only one full length biography in English (with almost

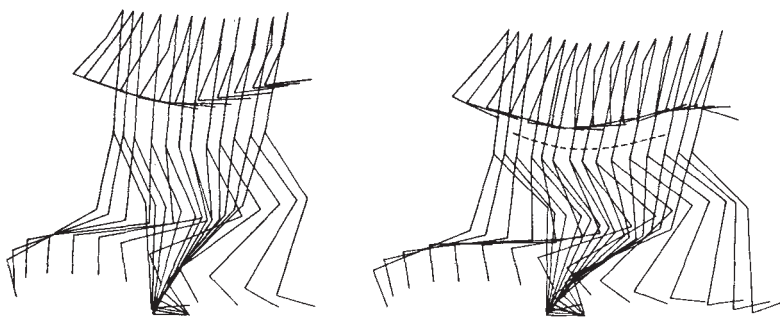
no discussion of her mathematics), A.H. Koblitz's *A Convergence of Lives* (Birkhäuser, 1983).

By describing in some detail the investigations of her predecessors, the author has quite correctly presented Kovalevskaya's achievements in a proper historical context. For example, in discussing one of her best known results — usually referred to as the Cauchy–Kovalevskaya theorem — he distinguishes between the modern textbook version (that it is an existence and uniqueness proof) and what Kovalevskaya, working within the Weierstrassian tradition, had set out to do, namely to demonstrate how analytic functions could be defined by means of formal power series arising as solutions of partial differential equations. Cooke has widely supplemented the description of Kovalevskaya's results with appendices containing technical details and has provided suitable “translations” of the original results into contemporary language. However, despite the author's intention to write a book for non-specialists (p.vii), I do not believe that the more technical sections will be accessible to readers who have not (at the very least) completed a course in complex function theory.

Today Kovalevskaya's achievements have taken on something of a mythical quality, and she has been elevated to a prominent position in the feminist pantheon. Thus a valuable part of this volume contains assessments of her work made by her contemporaries, H. Poincaré, A.A. Markov, V. Volterra, G. Mittag-Leffler and (in 1926) F. Klein. Cooke generally concurs and concludes that she was

a competent, creative mathematician who produced some valuable work and a few works of minor importance, making an occasional mistake in the process ... she would not seem quite so remarkable if she had lived a century later, when no one is surprised to see a competent woman mathematician [pp.178–179]. □

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Run to ground — stick figures traced from movie films showing normal running (left), and a perturbed form of running (Groucho running) in which the aerial phase of the gait and the bumpiness of the ride are reduced but the metabolic cost is higher. The illustration is taken from a paper in *Design and Performance of Muscular Systems*, recently published by The Company of Biologists. Distributor is The Biochemical Society, price is £25, \$60.

Burning conditions

D. B. Spalding

The Mathematical Theory of Combustion and Explosions. By Ya. B. Zeldovich, G.I. Barenblatt, V.B. Librovich and G.M. Makhviladze. *Consultants Bureau (Plenum)*: 1985. Pp.597. \$95, £90.25.

IN ESSENCE, the mathematical theory of combustion is simple: molecules interact, and are created or destroyed, as a consequence of the reactions studied by chemical kineticists; these sources and sinks give rise to concentration and temperature gradients which engender diffusional fluxes, of the kind represented by the laws of Fick and Fourier; simultaneously, either because of the density changes or of conditions imposed at the boundaries, the products and reactants are set in motion in a manner governed by the Navier-Stokes equations; the high temperatures give rise to radiative transport of energy in accordance with the classical laws; and the interchanges of mass, momentum and energy with the surroundings can also be expressed quantitatively.

A given combustion phenomenon can therefore be represented by an established set of simultaneous partial differential equations, complicated by the fact that many of the included variables (for example density, concentrations, temperature) are linked by known algebraic relations expressing the nature of the materials in question. Once the initial and boundary conditions have been fully worked out, the problem in question is then ready for solution.

Of course, the non-linearity of the equations, and the multi-material, multi-dimensional character of most combustion phenomena, demand the use of numerical methods and of powerful digital computers. A consequent danger is that the employment of such formidable apparatus will so distract and overburden the theorist that he fails to achieve that perception of interrelationships, and ability to anticipate outcomes, which we call "understanding".

Any combustion specialist who wishes to strengthen his understanding will do well to study the present book. Soviet scientists have done much of the pioneering research on this subject, particularly in the formulation of concepts and provision of explanations of major phenomena. The senior author has been especially prominent (with Semenov, Frank-Kamenetskii, Shchelkin, Vulis and others) while his co-authors too have themselves made notable contributions.

That pioneering work was performed before electronic digital computers were available, for which reason the mathematical formulations had to be simplified; but it was conducted with realism and intelligence, and without extremism. Just

enough of the physics was retained (and especially the steep temperature dependences which give rise to nonlinearity) for the important phenomena of ignition, propagation, instability and extinction to be understood; and simple approximate methods of solution were devised for the resulting equations.

The book retains many of the virtues of the early works. But although it pays some attention to more recent trends and pre-occupations, it does not follow them very far. The reader will therefore find little about theories of turbulent combustion; and recent advances in large-scale computer simulations of flames are not mentioned.

The original Russian edition of the book appeared in 1980. Its lucidity has been well preserved in the English translation and it can be read with both pleasure and advantage by combustion scientists in the Western world, several of whom will find that ideas which they have attributed to themselves or their contemporaries were already thoroughly understood a generation ago. The authors have, incidentally, addressed a graceful preface to their Western readers, while their introduction, which is the same as that of the Russian edition, reveals their full awareness that combustion is a truly international science. □

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Fit for survival

John Hearn

Reproduction in Mammals, 2nd Edn. Book 4, Reproductive Fitness. Edited by C.R. Austin and R.V. Short. *Cambridge University Press*: 1985. Pp.241. Hbk £22.50, \$39.50; pbk £8.95, \$14.95.

THE success or failure of reproduction is crucial to survival or extinction, and thus of immense evolutionary importance. Knowledge of the process is also of great consequence in the management and conservation of species diversity in an ever more crowded world. This will continue to be a central question during the next 50 years, over which the world's human population will double to nearly nine thousand million. Much of the increase will take place in developing countries that now provide the remaining homelands of many exotic species.

Our current understanding of reproductive strategies, species variations, the genetic, behavioural and immunological factors in reproductive fitness, and the influence of senescence, is well presented in this volume, the updated version of Book 4 of the first edition of *Reproduction in*

Mammals (published in 1972 under the title *Reproductive Patterns*). It is virtually a different book: two authors of the original complement remain and there are now seven chapters instead of five. All of the contributions are valuable, but among the newcomers I particularly enjoyed the chapters on genetics (Roger Land), environment (Brian Follett) and reproductive strategies (May and Rubenstein). Revision of the series as a whole continues next year with the appearance of the fifth volume, to be entitled *Manipulating Reproduction*.

While we are rapidly improving our appreciation of general principles, we are still desperately ignorant of precise details of the basic factors that limit survival of individual species. Among mammals alone we have an adequate understanding of fewer than 50 of the 4,000 species, including those on which our agriculture and biomedical sciences are based, and our domestic pets. It is ironic that this knowledge has contributed enormously to the success of our own species in helping us to overcome all natural environmental constraints, but in consequence threatens the survival of most other species. There is a pressing need to define the essential requirements of key species, so that conservation and management policies for the shrinking wildernesses genuinely meet the needs of these species and of others that share the same territory. A hard, quantifiable, scientific approach, at the fundamental level achieved in this book, is critical for the future of animals, including man.

To quote from Roger Short's contribution (Chapter 2, on species differences):

Readers ... will have been struck by the enormous diversity of reproductive mechanisms across mammalian species. About the only common factor is that, somewhere along the line, a spermatozoon meets an egg and so a new individual is formed. Everything else is subject to enormous variability, so that generalisations become impossible. This may act as a deterrent to the faint hearted, who are baffled by complexity; in reality it should be seen as an exciting challenge, encouraging us to probe deeper for the reasons underlying the diversity.

The strength of this book, and of the series of which it is part, is that a bewildering complexity and variability of species, physiological mechanisms and scientific disciplines is made readable, understandable and exciting. The contents are right up to date and point the way to further research development. While the book is aimed primarily at an undergraduate audience, research workers and lecturers will also find it invaluable. As a definitive text in a fast-moving field, I commend it as required reading for those interested in reproductive biology. □

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Charting the decline in British science

from John Irvine, Ben Martin, Tim Peacock and Roy Turner

Recent data on the comparative scientific performance of different nations suggest that the decline in British basic science apparent during the 1970s began to level off at the start of the 1980s. However, rather than heralding the beginning of a revival, the evidence suggests that this is merely a temporary halt to the long-term slide.

It is now almost conventional wisdom that British science is in a state of crisis. Recent leading articles in *Nature*, for example, have had a tone of desperation, with headlines such as "University paymasters discredited — the British government's latest policy document on higher education is a disgrace", "More crisis for UK research", and "Parliament's chance to act". A similar, if more restrained, note of despair has been sounded by the Advisory Board for the Research Councils (ABRC) in its latest report, *Science and Public Expenditure 1985*, which was submitted in June to the Secretary of State for Education and Science. In unambiguous terms, this stated that, "We believe that Parliament, the Government and the country as a whole are complacent about the current financial straits of the dual support system" (for financing research in universities).

In these and other analyses of the situation now confronting British science, most attention has been focused on inadequacies of the funds and other inputs to research. Largely for lack of data, less has been said about the effects of financial constraints on the outputs from science. We attempted to remedy this last year in a study for the ABRC which made use of a range of research output indicators (world shares of publications and citations, and figures on Nobel Prizes and patents), seeking thus to provide systematic information on long-term trends in British science.

The evidence suggested that, during the latter half of the 1970s, Britain's basic science declined appreciably, both in regard to relative output of publications and in their impact on the international research community. It was argued that the government may not be providing sufficient support for research (or at least not concentrating it in the right places) to ensure the successful development of new core technologies vital to the long-term survival of British industry.

The subsequent public interest in the results of the study (see *New Scientist*, 1429, pp.25–29, November 1984) apparently reflects a growing realization that, despite all the limitations of scientific output indicators, it will be necessary to make much greater use of systematic information on research performance in for-

mulating a coherent national research policy if Britain is to ensure that the scientific needs of new technology-based industries are met. Trying to steer publicly funded research using only the mechanism of scientific peer-review (or "the informed prejudices of wise men", as it has been aptly described), while totally ignoring quantitative input and output indicators, is somewhat like trying to run the economy using only the judgements of a committee of senior industrialists.

In what follows, we update the figures on Britain's performance in basic research to the end of 1982, and provide more detailed data on trends in some of the main fields of science. The work involved was supported by a consortium of British learned societies who have set up an informal working party (Ad Hoc Working Group for Research Funding, AGREF) under the chairmanship of Professor J. Lamb (St Andrews University, UK) to examine government support for science.

Measuring performance

Perhaps the most readily accessible data on national research outputs are the publication and citation indicators contained in the National Science Foundation's *Science Literature Indicators Data Base*. This is compiled by CHI Research on behalf of NSF for use in the biennial US *Science Indicators* report, and is based on the *Science Citation Index* produced by the Institute for Scientific Information (ISI). (This database is the source of the material pre-

sented in Tables 1–7, and a copy of it is held at the Science Policy Research Unit, University of Sussex.) The primary dataset covers some 2,000 of the world's leading scientific journals over the decade 1973–82, and contains figures on numbers of publications and citations for more than 190 countries and geographical regions, broken down into eight main scientific fields (for example, physics) and, in the case of the publication data, into approximately 100 subfields (for example, optics).

The use of such bibliometric data to indicate research output in basic science entails a number of assumptions. One is that most new scientific knowledge is eventually encapsulated in the form of research articles in learned journals. If this is the case, it follows that aggregate figures on the number of British publications in specified fields compared with those from other countries should give some indication of Britain's relative scientific output and, more importantly, of trends over time. A second assumption is that citation counts (that is, the number of times papers are subsequently referred to by other scientists) can be used to gauge the impact of research findings on the scientific community.

At the outset, we list three important caveats. First, the ISI data are appreciably biased in favour of English-language journals, which means that the scientific contributions of West Germany, France, Japan and, in particular, the Soviet Union

Table 1 UK world share of publications by field of research, 1973–82

Field	Percentage of total					Av. ann. % change 1973–80	Av. ann. % change 1980–82	Total % change 1973–82
	1973	1975	1978	1980	1982			
Biology	9.5	10.7	10.8	10.2	9.9	+1.1%	–1.5%	+4%
Biomedical research	9.4	9.6	8.8	8.4	8.4	–1.5%	0.0%	–10%
Chemistry	7.8	8.3	7.0	6.8	6.9	–1.8%	+0.7%	–12%
Clinical medicine	10.4	10.4	9.5	9.3	9.4	–1.5%	+0.5%	–9%
Earth and space sciences	8.1	9.5	8.9	9.5	8.9	+2.5%	–3.2%	+10%
Engineering and technology	10.7	11.0	9.4	9.5	8.6	–1.6%	–4.7%	–20%
Mathematics	7.4	8.2	7.0	7.0	7.0	–0.8%	0.0%	–5%
Physics	7.7	7.4	6.3	5.9	6.1	–3.3%	+1.7%	–21%
All fields combined	9.2	9.5	8.6	8.3	8.3	–1.4%	–0.1%	–10%

are underestimated. Second, the CHI/NSF trend data are based on an essentially constant set of journals established in 1973; papers published in entirely new journals are not covered, with the result that newly emerging or fast growing areas of science may be under-represented. This disadvantage is, however, offset by the fact that more reliable analyses of trends over time can be undertaken. (Since 1981, CHI Research has in fact compiled an alternative data series based on the 3,000 journals scanned by ISI in that year, but their analysis suggests that this larger database may be more, rather than less, biased in favour of English-language countries.) Third, since CHI Research assigns articles to fields and subfields according to the journals in which they are published, some problems can arise in relation to smaller specialities, in particular those of an interdisciplinary nature where researchers publish in a great variety of journals.

Britain's performance

Table 1 contains data on changes over the decade up to 1982 in Britain's world share of publications broken down by field of research. While there has been a decline of 10 per cent in overall world share (down from 9.2 per cent to 8.3 per cent), the picture is uneven. Physics and engineering have suffered significant decreases of 21 per cent and 20 per cent respectively, but biology has marginally increased its world share by 4 per cent over the 10 years. More encouraging, at least at first sight, is that the downward trend evident since 1975 seems almost to have levelled off in the two years since 1980, with physics, chemistry and clinical medicine apparently registering small increases. We shall return to the question of whether this halt in the downward trend is likely to continue or is merely a temporary phenomenon.

On the impact of British work on the international research community, a rather less optimistic picture emerges from the citation figures in Table 2. It can be seen that the decline which began in the 1970s has continued into the 1980s — the world share of citations for all fields combined has decreased by 15 per cent since 1973, with biomedical research, engineering and physics all recording falls of over 20 per cent. (Figures based on the 1981 journal-set are relatively consistent with these values.) Furthermore, although there had been a slight decrease in the rate of decline from 1.9 per cent per annum in the 1970s to 1.2 per cent in the early 1980s, there is little sign in the citation figures of the levelling off seen in the world share of publications.

When the figures for Britain are set against those for the other main industrialized nations, the implications of the long-term decline in Britain's basic science become all too apparent. Table 3 gives a breakdown by field of percentage changes in national shares of world publications

Table 2 UK world share of citations by field of research, 1973–82

Field	Percentage of total					Av. ann. % change 1973–80	Av. ann. % change 1980–82	Total % change 1973–82
	1973	1975	1978	1980	1982			
Biology	11.9	12.5	12.8	12.0	11.4	+0.1%	-2.5%	-4%
Biomedical research	11.7	10.4	9.0	8.5	8.4	-0.6%	-3.9%	-28%
Chemistry	11.2	10.2	11.5	10.3	9.8	-1.1%	-2.4%	-12%
Clinical medicine	12.8	12.8	11.5	11.3	11.5	-1.7%	+0.9%	-11%
Earth and space sciences	9.4	9.9	8.9	9.3	8.9	-0.2%	-2.2%	-5%
Engineering and technology	11.5	12.1	10.9	9.9	9.0	-2.0%	-4.5%	-21%
Mathematics	8.7	9.4	7.3	7.8	7.4	-1.5%	-2.6%	-15%
Physics	7.6	7.8	6.1	6.7	5.9	-1.7%	-6.0%	-22%
All fields combined	11.2	10.9	10.2	9.7	9.5	-1.9%	-1.2%	-15%

Table 3 Percentage changes in world publication share of major industrial countries by field, 1973–82

Field	Canada	France	FRG	Japan	UK	USA	USSR	Rest of world
Biology	+6%	-30%	-10%	+24%	+4%	-5%	-7%	+8%
Biomedical research	-10%	-27%	+7%	+64%	-10%	+5%	-7%	-5%
Chemistry	-17%	-17%	+11%	+23%	-12%	-6%	-11%	+12%
Clinical medicine	+4%	-6%	-13%	+62%	-9%	-2%	-17%	+5%
Earth and space sciences	+5%	-4%	+46%	+4%	+10%	-8%	-9%	+12%
Engineering and technology	-15%	+31%	0%	+45%	-20%	-1%	-31%	+22%
Mathematics	-24%	0%	+15%	+52%	-5%	-19%	+74%	+31%
Physics	-19%	+7%	+28%	+38%	-21%	-9%	-16%	+20%
All fields combined	-8%	-9%	+2%	+40%	-10%	-3%	-15%	+9%

Table 4 Percentage changes in world citation share of major industrial countries by field, 1973–82

Field	Canada	France	FRG	Japan	UK	USA	USSR	Rest of world
Biology	+13%	-2%	+13%	+32%	-4%	-5%	-45%	+6%
Biomedical research	-9%	+10%	+48%	+83%	-28%	-4%	-6%	+3%
Chemistry	-14%	+3%	+4%	+59%	-12%	-9%	-13%	+11%
Clinical medicine	+2%	+17%	+3%	+84%	-11%	-4%	-27%	+6%
Earth and space sciences	-6%	+9%	+66%	+35%	-5%	-3%	-34%	+12%
Engineering and technology	-23%	+8%	-11%	+91%	-21%	+4%	-51%	+30%
Mathematics	-10%	+19%	+3%	+53%	-15%	-13%	+5%	+42%
Physics	-23%	+13%	+35%	+69%	-22%	-15%	-23%	+37%
All fields combined	-7%	+8%	+14%	+65%	-15%	-5%	-27%	+11%

over the period 1973–82. Japan has increased its overall share by no less than 40 per cent and the rest of the world by 9 per cent; the main losers have been Britain (-10 per cent), Canada (-8 per cent), France (-9 per cent) and the Soviet Union (-15 per cent), although in the last two cases there are likely to be appreciable biases in the statistics since French and Soviet scientists may well be preferring to publish more in domestic journals not scanned by ISI.

Such an effect would also give rise to an element of bias (probably rather smaller) in the figures on changes in national shares

of world citations given in Table 4. These suggest that Japanese researchers in all eight fields have markedly increased their impact on the international scientific community, with an overall citation share that has risen by 65 per cent during the 10 years to 1982. Similarly, West Germany and the rest of the world increased their overall shares by more than 10 per cent, with France also recording a significant increase (8 per cent). Britain and the Soviet Union have again been the main losers, with declines of 15 per cent and 27 per cent respectively. One interesting feature of the table is that Japan's largest gains in

Table 5 Strengths and weaknesses in British science: share of world publications, 1982

Weaker areas: < 7 per cent of world share		Stronger areas: > 11 per cent of world share	
	% Share		% Share
Clinical medicine-pharmacy	1.2	Anaesthesiology	18.4
General chemistry	3.7	Pathology	15.6
General physics	4.3	Tropical medicine	15.1
Metallurgy	4.6	Ecology	14.6
Nuclear and particle physics	4.6	Veterinary medicine	13.3
Optics	4.6	General and internal medical research	13.0
Polymer research	5.5	Inorganic and nuclear chemistry	13.0
Oceanography and limnology	5.6	Probability and statistics	13.0
General mathematics	5.7	Gastroenterology	12.9
Ophthalmology	5.7	Dermatology and venereal disease	12.7
Radiology and nuclear medicine	5.7	Electrical engineering and electronics	12.7
Chemical engineering	5.9	Hygiene and public health	12.5
Nuclear technology	6.0	Dentistry	12.2
Solid-state physics	6.0	Microbiology	12.0
Applied physics	6.1	Geology	11.9
Agricultural and food science	6.4	Haematology	11.8
Cardiovascular research	6.7	Botany	11.3
General biomedical research	6.8	Organic chemistry	11.3
Analytical chemistry	6.9		
Surgery	6.9		

Table 6 Major recent trends in British share of world scientific publications, 1977-82

Increase of ≥ 15 per cent in world share, 1977-82		Decline of ≥ 15 per cent in world share	
	% Increase		% Decrease
Entomology	70	Metallurgy	34
Computer technology	61	Clinical medicine-pharmacy	32
Dentistry	17	Materials science	31
Gastroenterology	17	Chemical engineering	26
Geology	16	Tropical medicine	26
Immunology	16	Nuclear and particle physics	26
Haematology	15	Cell biology, cytology and histology	24
		Polymer research	24
		Veterinary medicine	23
		General physics	22
		Electrical engineering and electronics	21
		Applied chemistry	19
		Ecology	19
		Applied physics	17
		Pathology	17
		Botany	16
		Dermatology and venereal disease	16
		Nutrition and dietetics	16
		Endocrinology	15

world citation share have tended to be in fields where Britain has declined most, biomedical research, physics and engineering in particular.

One finding from our previous analysis of earlier NSF/CHI data was that the weakest parts of British basic science are heavily concentrated in areas with strategic technological importance for the future. This finding is reinforced by the updated statistics reported in Table 5. This table lists those scientific specialities in which Britain had a world publication share of more than 11 per cent or less than 7 per cent in 1982. One can see that the areas of strength are mainly in medical and biological specialities, although organic and inorganic chemistry, and electrical engineering and electronics are also quite strong. In contrast, the weaker areas are generally in more applied scientific and engineering specialities such as metallur-

gy, optics, polymer research, chemical engineering and applied physics. What is particularly alarming is the continuing rapid decline in several of these areas; in just two years since 1980, Britain's world publication share in optics dropped from 5.5 per cent to 4.6 per cent, in polymer research from 6.3 to 5.5 per cent and in chemical engineering from 6.7 to 5.9 per cent.

More systematic evidence on recent trends is contained in Table 6, which shows the research areas where Britain has either increased or decreased its world publication share by over 15 per cent in the five years up to 1982. With the exception of computer technology, the overall picture is again one of major declines in areas of science likely to prove strategically important in the future — for example, materials science, applied chemistry and physics, and electrical engineering and

electronics.

We have argued elsewhere that companies in both traditional and new industrial sectors are becoming increasingly dependent on the results of research for maintaining innovative activity and international competitiveness (see *Foresight in Science: Picking the Winners*, Pinter, London, 1984). The figures on recent trends in British science, especially those for more applied scientific and engineering research, thus give major cause for concern, particularly when set in the context of the dramatic fall that has already occurred over the last decade or so in the country's relative technological standing. One indication of the latter is the British share of foreign patents registered in the United States. OTAF data shows that, in the past 20 years, this has declined from around 20 per cent to 8 per cent, while Japan's share has, over the same period, risen from under 10 per cent to over 35 per cent, and West Germany's has remained broadly constant at about 25 per cent, as has that of France at 8 per cent. Clearly, if Britain is to compete more effectively in the international technology market, one important precondition will be an increase in the strength of its underlying research infrastructure.

Optimism

It is therefore worth looking more closely at what has happened since 1980 to determine whether there is any evidence that the long-term decline in British science shows any sign of being reversed. As noted earlier, the figures relating to the British world share of publications do initially seem to provide some grounds for optimism. From the figures in Table 1, one can see that, while the overall share fell from a peak of 9.5 per cent in 1975 to 8.3 per cent in 1980 (a fall of 14.5 per cent in five years or nearly 3 per cent per annum), the British share has remained approximately constant since 1980. Does this herald a renaissance in British science?

There are several possible explanations for this levelling-off in the relative output of British publications since 1980. One is that British scientists, perhaps imbued with a new sense of economic realism engendered by the policies of the Conservative government after its election in 1979, realized the need to increase their productivity and began to publish more research articles in international journals.

In our view, however, a more plausible explanation relates to the funds received by science. Most British academic research is supported by grants from the five research councils with indirect support provided by the University Grants Committee. Figures on research council expenditures in constant prices (calculated using the official EEC research and development price deflator) for 1970 onwards have been plotted graphically in Fig. 1, the expenditure for each year being

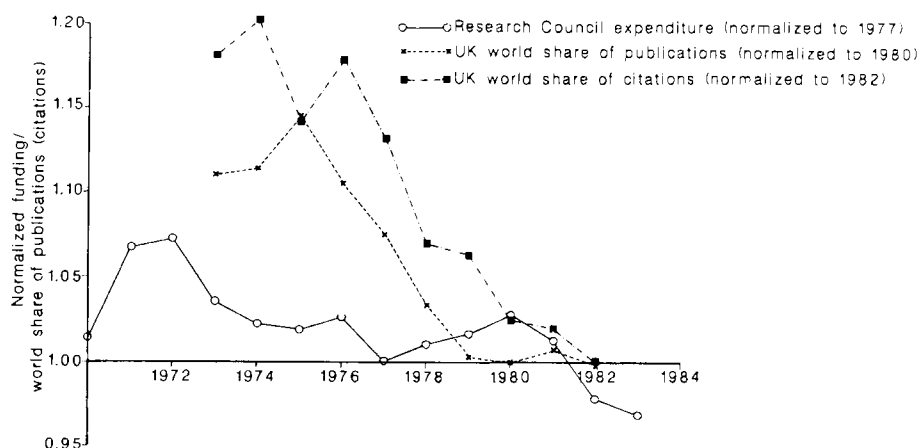


Fig. 1 Association between research council expenditure and UK world share of publications/citations. Source: Tables 1 and 2 on pp.6 and 19 of Butler, E. B. (ed.) *Research and Development in the UK* (Technical Change Centre, London, 1984), which are in turn based on figures published by the Central Statistical Office of the United Kingdom.

expressed as a ratio of that in 1977. As can be seen, research council expenditure grew in real terms between 1970 and 1972, before declining gradually to a minimum in 1977. In the three years up to 1980, expenditure grew slowly but steadily, but since then has fallen sharply in real terms.

Figure 1 also plots the changes in Britain's world share of publications and citations. In the case of publications, there was a peak in 1975 (three years after the peak in funding), but then a rapid fall reaching a minimum in 1980 (again three years after the trough in funding); 1981 registered a modest increase, while in 1982 the British world share began to fall again. There is thus a certain similarity between the trends over time in funding and national share of publications. It would appear that the 6 per cent increase in funding between 1970 and 1972 was reflected in the 3 per cent increase in Britain's share of publications between 1973

and 1975, while the 7 per cent decline in funds in the five years up to 1977 was associated with the sharp drop of 14 per cent in publication share between 1975 and 1980 (see Table 7). In other words, there seems to be a time lag of approximately three years between changes in expenditure and publication share. (This is the reason why the figures on publications have been normalized to 1980 compared with 1977 for funding.) Similarly, there is some suggestion of a time lag be-

tween publication and citation world shares, with the rapid fall in the former between 1975 and 1979 apparently being mirrored in a similarly sharp decline in the latter between 1976 and 1980. If there is indeed an association between research expenditure and the British share of world publications, this would suggest that the levelling off in publication output was largely the result of the 3 per cent increase in research expenditure between 1977 and 1980. Further, if such an association were to continue to hold in the future, one would expect the drop in funding since 1980 to be followed some three years later by a sharp decline in the UK share of publications. It will be interesting to see whether this is actually the case when the CHI/NSF data for 1983-84 become available in 1986. Furthermore, according to the ABRC's latest report, research council funds have continued to fall in real terms since 1983 (by perhaps 1 per cent per annum) and, on the basis of government estimates, will probably continue to do so over the rest of the decade. If the past is any guide, it seems that the UK world share of publications may decline at a somewhat faster rate over the foreseeable future.

Much more research is obviously needed to establish the existence of a direct relationship between funding and publication output and also to explore the reasons behind the fall in the British share of citations. (One reason, for example, may be the widely reported shortage of front-line research equipment in universities and research council laboratories.) Nevertheless, it is clear that one can take little comfort from what has happened since 1980 — the evidence suggests that the levelling-off in the decline of the UK world share of publications between 1980 and 1982 will prove only a very temporary phenomenon.

Over the last year, there has been a considerable growth in concern in Britain with the state of science. At the invitation

of ABRC, the Royal Society is undertaking a study of the health of British science which may provide some evidence on the extent to which the pessimistic conclusions following from bibliometric data of the type presented above are consistent with the views of the scientific community. The Secretary of State for Education and Science is, according to his speech during the 14 June House of Commons debate on science policy, awaiting the completion of that study before making his judgement about whether the quality of science in Britain has declined. Others, however, are already extremely concerned. For example, John Harvey-Jones, chairman of ICI, had this to say about a thorough review by the company of where its next science-based innovations would come from: "I think there is some evidence that the United Kingdom is sliding in the world pecking order. The evidence is still tenuous, but it has led us to start increasing our links with overseas universities that we see as centres of excellence. . . . If our suspicions are correct that the United Kingdom is no longer up at the front as it was, then that presents, in the long haul, very dangerous problems for us." (Quoted in *The Guardian*, 19th February 1985, p.24.) As one of the Members of Parliament who spoke earlier in the parliamentary debate warned, "this may be the last chance . . . to stop the catastrophe that we face as a scientific and educational nation".

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Table 7 Association between research council expenditure and UK world share of publications

Research council expenditure		UK share of publications	
1970-72	+6%	1973-75	+3%
1972-77	-7%	1975-80	-14%
1977-80	+3%	1980-82*	0%
1980-83	-6%	1983-86	?

* 1983 figure not yet available

and 1975, while the 7 per cent decline in funds in the five years up to 1977 was associated with the sharp drop of 14 per cent in publication share between 1975 and 1980 (see Table 7). In other words, there seems to be a time lag of approximately three years between changes in expenditure and publication share. (This is the reason why the figures on publications have been normalized to 1980 compared with 1977 for funding.) Similarly, there is some suggestion of a time lag be-

A 150,000-year climatic record from Antarctic ice

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During much of the Quaternary, the Earth's climate has undergone drastic changes most notably successive glacial and interglacial episodes. The past 150 kyr includes such a climatic cycle: the last interglacial, the last glacial and the present holocene interglacial. A new climatic-time series for this period has been obtained using $\delta^{18}\text{O}$ data from an Antarctic ice core.

ALTHOUGH continuous records of climatic and environmental conditions extending beyond 150 kyr are preserved in polar ice sheets, a reliable time series covering the whole of this climatic cycle has not yet been derived from ice cores. This results from logistic reasons, the favourable sites being located in the central regions of Antarctica and Greenland, and the technical difficulty of deep drilling.

The Quaternary has undergone successive glacial and interglacial episodes¹⁻³. Since the first Camp Century drilling in north-west Greenland⁴, five deep-ice cores reaching back to the last glacial have been recovered in Antarctica at Byrd⁵, Vostok⁶ and Dome C⁷ and in Greenland at Dye 3⁸. They have been extensively studied for their oxygen-18 content (denoted $\delta^{18}\text{O}$ expressed with respect to SMOW) and their chemical impurities, which provide valuable information about the Earth's palaeoclimate and palaeoenvironment (see refs 9, 10).

Of these cores, Camp Century seems to extend back to the last interglacial. As it was drilled down to the bedrock, however, this last interglacial ice is confined to the last few metres of the core, because of the thinning of the ice layers during their sinking in the ice sheets. The complexity of the flow lines makes it difficult to date ice cores reaching the bedrock by flow modelling only. Indirect dating techniques, based on the comparison of $\delta^{18}\text{O}$ profiles in ice cores and in deep-sea cores have recently been used⁷⁻⁹ for this purpose. A new Camp Century timescale was proposed which confirms the presence of last interglacial ice and, accordingly, indicates that the deepest part of the Dye 3 ice core was probably deposited during this period⁹. This is possibly also the case for the Devon Island core drilled through a shallow ice cap in the Canadian Arctic¹¹.

We have determined a new climatic-time series obtained from $\delta^{18}\text{O}$ analysis of a 2,083-m ice core drilled at Vostok Station by the Soviet Antarctic Expeditions. The site is located on the high Antarctic Plateau, where the ice thickness is ~3,700 m. The isotope profile covers the last 150 kyr, back to the ice age which preceded the last interglacial, and has essentially been undisturbed by flow conditions.

Vostok ice core

The Soviet Antarctic station of Vostok is located in East Antarctica (78°28' S and 106°48' E) at an altitude of 3,488 m (mean annual pressure 624 mbar; mean annual temperature -55.5°C). The present snow accumulation is between 2.2 and 2.5 g cm⁻² yr⁻¹ (ref. 12) as determined independently from a profile of artificial β activity¹³ and detailed stake measurements¹⁴.

The first series of drilling was performed down to 950 m in successive steps between 1970 and 1974. This 950-m core covers all the Holocene and part of the last glacial periods as shown by the $\delta^{18}\text{O}$ profiles obtained by Barkov and co-workers^{6,15,16}. In 1980, the drilling of a second deep hole was started which

has reached a depth of 2,083 m. The $\delta^{18}\text{O}$ measurements were completed down to 1,412 m (refs 17, 18), a depth which would correspond, from a preliminary dating, to 115 kyr BP (ref. 19).

The collaboration between Soviet and French scientists which centred around the study of this new 2,083-m Vostok ice core was initiated in 1982. Within this framework, sampling was done in the field during the 1982-83 Austral summer and pieces of ice core were sent to Grenoble. The core analysis now in progress in various Soviet and French laboratories concerns, in particular, the chemical analysis of impurities and gas bubbles including CO₂.

$\delta^{18}\text{O}$ profile

Sampling of ice for isotope analysis was done in the field (1982-83 Austral summer) by cutting a continuous slice from the length of the ice after careful cleaning. Sampling was performed on 1.5-2 m ice increments. Samples were sent in solid form to Grenoble and then melted before isotope analysis in Saclay. Two independent series of samples were obtained: (1) a discontinuous series, duplicated to check reproducibility, with one sample taken each 25 m from the surface down to the bottom of the core; (2) a continuous series between 1,406 and 2,083 m.

Oxygen-18 and deuterium determinations were simultaneously performed on all the samples. Here we will discuss the $\delta^{18}\text{O}$ results. The deuterium and deuterium-excess²⁰ results will be discussed elsewhere. The results, obtained with an accuracy of $\pm 0.15\%$ (w.r.t. SMOW), are shown in Fig. 1 as a function of the sample depth. The two sets of samples from the discontinuous series are in good agreement all along the core and their average value at each level is given. For the sake of discussion a continuous line was drawn between each point. There is excellent agreement between the continuous and discontinuous series over all the common parts indicating that the 25-m sampling interval is fine enough to extract reliable palaeoclimatic information. On the other hand, the present data are not detailed enough to document for the Southern Hemisphere such features as the abrupt environmental changes revealed in the Dye 3 ice core between 30 and 40 kyr BP (refs 9, 21).

Down to 1,400 m, the basic features of the $\delta^{18}\text{O}$ curve (Fig. 1) are similar to previously published Vostok $\delta^{18}\text{O}$ profiles^{17,18}, with the last glacial maximum around 400 m, and a $\delta^{18}\text{O}$ shift between glacial and post-glacial conditions of ~5‰. However, there is a systematic shift towards lower values for the present set of data of ~3‰. Such a shift has already been noticed by Dansgaard *et al.*²².

The $\delta^{18}\text{O}$ profile presented here gives a complete picture of the last climatic cycle from the end of the previous glaciation. In particular, the last interglacial with the highest $\delta^{18}\text{O}$ values around 1,825 m can now be examined in detail. For a further

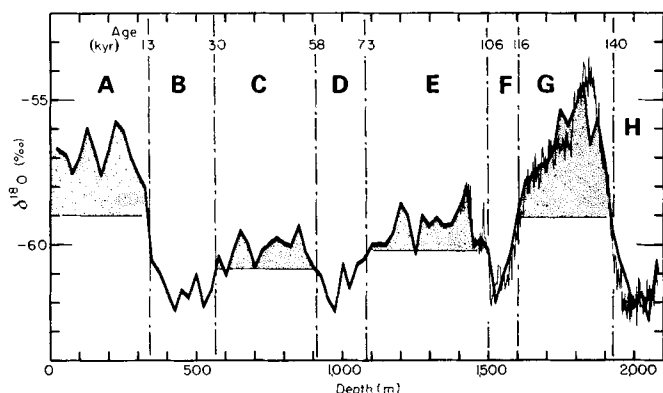


Fig. 1 Oxygen-18 versus depth in the Vostok ice with the definition of the successive stages and indication of the ages corresponding to the limits between these stages. The thick line is drawn from discontinuous data (one for each 25 m), the thin line corresponds to a continuous sampling between 1,400 and 2,083 m. For explanation of A-H, see text.

description of this curve and comparison with other palaeoclimatic profiles, we will designate the successive warm and cold stages consecutively from the top of the record downwards. A striking feature of the profile is the existence of four very well-marked cold periods (that is, low $\delta^{18}\text{O}$) around 425, 975, 1,525 and 2,050 m with practically the same $\delta^{18}\text{O}$ values of around -62‰ . This makes the definition of the successive stages shown on Fig. 1 relatively straightforward. Starting from the top, the warm periods (hatched areas), are designated by A, C, E and G and the cold periods by B, D, F and H. Stages A and G have the isotopically richest values lying around -57 and -55‰ , respectively. From the solid curve (Fig. 1), one can note a sharp isotope transition ($\sim 2\text{‰}$) inside the G stage at a depth of 1,790 m. Stages E and C are relatively warm interstadials intermediate between full glacial and interglacial conditions.

Dates for the limits between the successive phases are indicated on the top of Fig. 1 (the dating method used is described below). Stage A corresponds to the present Holocene period. Stages B-F cover the last glacial. Stage G includes the peak of the last interglacial and stage H is the last part of the previous glacial.

Ice-core chronology

Many techniques, including isotope and chemical stratigraphies and flow modelling, have been used over the past 10 yr to establish dating of the Vostok ice^{16,19,23,24}. Further detailed isotope study showed that seasonal $\delta^{18}\text{O}$ variations are rapidly smoothed by diffusion²² indicating that reliable dating cannot be obtained from isotope stratigraphy. Unlike the Camp Century core, where the existence of interglacial ice relies on a comparison with deep-sea core isotope profiles, we propose to establish the timescale using an ice-flow model which is independent of other palaeoindicators.

There are two problems with this type of dating. First, for each depth the rate of accumulation at the time and in that area of origin of snow, $A(z)$, must be determined. Second, the thinning function, that is, the ratio of the thickness of a layer to its initial thickness, has to be calculated. We now describe our approach to these problems.

To estimate the past accumulation rate we adopt a procedure based on the observation that the present precipitation rate on the Antarctic plateau is strongly correlated with surface temperature. Robin²⁵ has suggested that this correlation exists because precipitation is governed by the amount of water vapour in the atmosphere circulating above the surface inversion layer. As discussed by Lorius *et al.*⁷ and Jouzel and Merlivat²⁶, there is a link between the temperature of formation of precipitation and its oxygen isotope ratio. Assuming that the same relationship has held in the past, it is thus possible to estimate past accumulation rates from the $\delta^{18}\text{O}$ profile of the ice. Raisbeck *et al.*²⁷ and

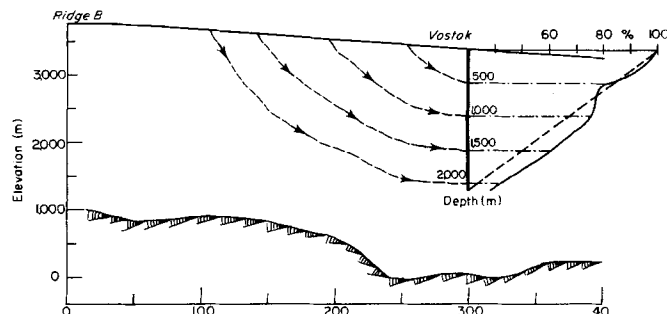


Fig. 2 Surface and bedrock topography along the Ridge B-Vostok axis (lower curve) and the thinning function (top right) expressed as a percentage of the initial thickness, with (solid line) or without (dotted line) taking into account this topography.

Yiou *et al.*²⁸ in this issue have noted that the ^{10}Be concentrations of ice at Dome C and Vostok are consistent with accumulation rates deduced by such procedures. We thus estimate the accumulation function in the following way. First, the temperature of formation of precipitation, T_F , is estimated along the profile as described by Lorius *et al.*¹⁰. Then, the accumulation rate, $A(z)$, is calculated from the product of its present value, $A(0)$, times the ratio of the derivatives of the saturation vapour pressure—with respect to $T_F(z)$ for the time of precipitation and with respect to $T_F(0)$ for the present conditions. This formulation is derived from a simplified unidimensional model neglecting the possible changes in circulation intensity above the area of precipitation.

The use of a two-dimensional glaciological model is well suited to the Vostok area as the ice flowlines are roughly parallel in this region of East Antarctica²⁹. The change of the ice thickness upstream of the drilling site is important because of the bedrock topography³⁰ (Fig. 2) and this has been taken into account when using the analytical model of Lliboutry³¹. Finally, ice-core dating is established using an iterative process. Starting from a plausible dating obtained from a simple one-dimensional model the ice origin is determined. Then, the thinning function is calculated and the ice redated all along the core. The process is repeated using the new dates as the initial conditions until the initial and final datings converge.

To illustrate the influence of the bedrock topography, the thinning functions with (Fig. 2, solid line) and without (Fig. 2, dotted line) taking into account this topography can be compared. This demonstrates the strong effect of bedrock topography between 500 and 1,000 m. For these calculations, ice velocities were approximated by their average values using mass continuity over the entire climatic cycle.

The results are presented in Fig. 3 for three values of the present accumulation. The annual accumulation (expressed with respect to its present value) is obviously correlated to the $\delta^{18}\text{O}$ profile with lower values during glacial periods as previously stated from Dome C ice-core studies^{7,27} and independently proposed at Vostok from ^{10}Be measurements²⁸.

From available data¹², an average value of $2.3 \text{ g cm}^{-2} \text{ yr}^{-1}$ was adopted for the present accumulation rate, giving ages of 98 kyr BP at 1,412 m (that is, significantly younger than the previously estimated¹⁹ 115 kyr BP) and of ~ 160 kyr BP for the bottom of the core.

This accumulation value, although uncertain given the very short period (< 10 yr) over which this parameter was determined, seems to be a reasonable average over the Holocene period. Our dating puts the end of the last climatic transition at around 10 kyr BP which compares well with the same event recorded in the Dome C East Antarctic core at ~ 10.5 kyr BP (ref. 7). The uncertainty on this value is thus $\sim 5\%$, a value which would give a lower limit for the relative dating uncertainty all along the core. Dating of the first part of the core is also indirectly supported by the existence of a dust content maximum between ~ 13 and 26 kyr BP (ref. 32), a period of aridity over Australia³³.

However, other sources of errors come from estimations of accumulation changes and, to a lesser degree, of the thinning function. Thus, the age of the bottom of the core is probably not known with a precision better than 10–15 kyr.

Palaeoclimatic information

The isotope content of polar snow is primarily governed by T_F , but it also depends^{9,10,34,35} on several other parameters such as the origin and dynamical history of the air masses and the microphysical processes leading to snow formation. A way of reconstructing the full, space-time variations of isotope contents of vapour and precipitation is to incorporate the isotope cycles in a general circulation model of the atmosphere (GCM)³⁶.

Until this new approach is fully developed, the climatic interpretation must be based on $\delta^{18}\text{O}$ -temperature relationships observed in modern precipitation and/or isotope models which give only a simple schematic overview of the dynamic history of the air masses. Although aware of the limitations, we are relatively confident that when the two methods are combined major temperature changes, such as those accompanying glacial-interglacial transitions, can be estimated. This is especially true for central East Antarctica for several reasons. First, the observed $\delta^{18}\text{O}$ -temperature relationship³⁷ is quite linear (correlation coefficient, $r=0.989$ for the -20 to -55°C interval) and its slope can be correctly explained on a theoretical basis applying a recently developed kinetic isotopic model²⁶. Second, the ice thickness has probably undergone only minor fluctuations and the ice origin can be reliably reconstructed. In addition, our confidence in a temperature reconstruction is reinforced in the Vostok core by the good agreement between accumulation changes derived by transforming the $\delta^{18}\text{O}$ profile into a temperature profile and those derived from ^{10}Be (ref. 28).

After correcting for the change in $\delta^{18}\text{O}$ of the oceanic water (maximum 1.6‰)³, this temperature reconstruction is carried out as in ref. 10. With this interpretation, the glacial-interglacial surface temperature shifts are $\sim 10^\circ\text{C}$ for the H-G transition ($\delta^{18}\text{O}$ shift of 6.5‰) and 8°C for the B-A transition ($\delta^{18}\text{O}$ shift of 5‰). C and E interstadials are around 2 and 4°C , respectively, warmer than full glacial conditions and the four cold stadials H, F, D and B are within $\sim 1^\circ\text{C}$. The peak of the last interglacial maximum is significantly warmer than the Holocene period. The difference is estimated to $\sim 3^\circ\text{C}$, 1°C of this being due to the formation of the interglacial ice 100 km further inland (Fig. 2). All these values do not take into account the possible variations in the ice-cap altitude. These corrections will be done, if necessary, from the total gas content which is under investigation. Preliminary data (D. Raynaud, personal communication) suggest that the isotope signal is essentially of climatic origin which is independently corroborated by some studies tending to demonstrate the relative stability of the East Antarctic ice sheet at the considered timescale^{38,39}.

Comparison with other ice cores

Concerning the transition between the last glacial maximum (around 18 kyr BP) and the Holocene periods (B-A in our terminology), the present results confirm the excellent agreement between the two East Antarctic sites, with a $\delta^{18}\text{O}$ shift, before corrections, of 5.4‰ at Dome C⁷ and of $\sim 5\%$ at Vostok, and to a lesser degree with the Byrd West Antarctic site (shift of 7‰)⁵. The inferred surface temperature change for Antarctica is ~ 8 – 10°C . These shifts agree well with those deduced for high southern latitudes from GCM ice-age modelling^{40,41}, although recent simulations predict lower temperature shifts^{42,43}. The isotope shift is also 7‰ in southern Greenland (Dye 3)^{8,9}. The higher values ($>10\%$) registered in northern Greenland and adjacent regions (Camp Century, Devon Island) can be partly attributed either to a change in the elevation of the ice formation⁴⁴ or to a more drastic climatic change in this area than in southern Greenland or in Antarctica⁹.

For the last glacial, a detailed comparison with other ice cores is difficult because only 7% of the Vostok core was analysed down to 1,400 m. In any case, it would be necessary to discuss

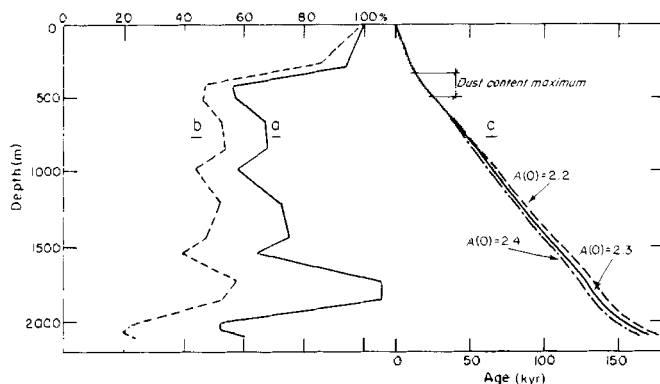


Fig. 3 a, Initial thickness of annual layers (accumulation rate $A(z)$); b, thickness of these annual layers in the core. Both a and b are expressed with respect to the present (accumulation rate $A(0)$); c, depth-age relationship for different values of $A(0)$ in $\text{g cm}^{-2} \text{yr}^{-1}$.

thoroughly the successive timescales derived for the Byrd, Camp Century and Dye 3 cores. Such a comparison will be undertaken in the near future when a continuous profile becomes available for the Vostok core.

The apparent time elapsed between the interglacial peak and the following $\delta^{18}\text{O}$ minimum is more than twice as long at Vostok than at Camp Century. This is not strictly an ice core feature and cannot be discussed further here (see below) as the Camp Century timescale is deduced from a comparison with the deep-sea record⁹. This shows the difficulty of applying such an indirect dating method.

Comparison with $\delta^{18}\text{O}$ in deep-sea cores

It is now widely accepted that $\delta^{18}\text{O}$ in benthic foraminifera primarily reflects ice-volume changes^{3,45,46}. Although the relationship can be distorted by many factors, the CLIMAP project members concluded that there is no compelling evidence to reject the first-order assumptions of isotope synchronicity or fidelity to the ice volume⁴⁵. Although varying in detail, all the cores give similar general trends^{3,47}, allowing a common definition of isotope stratigraphy. The period of interest here covers Emiliani's stages 1–6 (ref. 48), stage 5 being subdivided in 5a to 5e (ref. 49), as we have illustrated on Fig. 4e.

With the adopted Vostok dating, there is a good correspondence between stages 1–4 and Vostok stages A–D. Stage E covers 5a–5c and F corresponds to 5d. However, there is no clear correspondence between 5e and G representing the last interglacial in deep-sea and ice records, respectively (Fig. 4). There is a major difference in the duration of these two stages; 11 kyr for 5e and 24 kyr for G. Also, the last interglacial peak in the Vostok core is around 130 kyr BP whereas the corresponding ice-volume minimum in the deep-sea record is dated earlier at 122-kyr BP (ref. 45).

A doubling of the accumulation rate over this period would lead to a good correspondence between the 5e and G stages. While this possibility cannot be completely ruled out, there is no indication of such a drastic change in the precipitation regime. Part of the time-lag between the two peaks could be real (~ 3 kyr) as already observed between Southern Hemisphere ocean temperature and ice volume records but dating uncertainties (probably of ~ 10 kyr at this level) prevent a detailed discussion of this possible lag. On the other hand, the fact that ice stage G probably lasted longer than stage 5e is a key element for understanding the last interglacial climate and the initiation of the last ice age. A detailed interpretation is beyond the scope of the present study and would require knowledge of the exact timing between the temperature and ice volume signals. Recent measurements of $\delta^{18}\text{O}$ of air entrapped in ice cores could provide a way of correlating deep-sea and ice-cores $\delta^{18}\text{O}$ profiles and then establishing this timing⁵⁰.

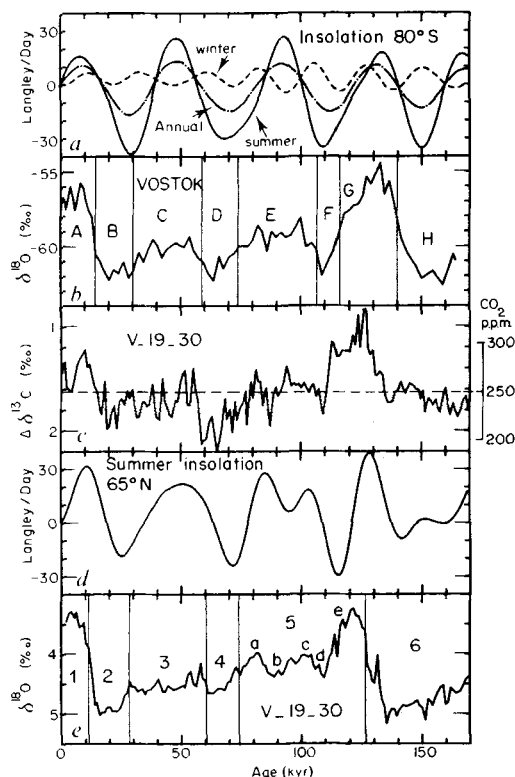


Fig. 4 Variation with time of: *a*, winter, summer and annual insolation at 80° S (ref. 59); *b*, $\delta^{18}\text{O}$ in the Vostok ice (this work); *c*, $\Delta\delta^{13}\text{C}$ in the V 19-30 deep-sea core⁶⁸ and indication of the CO_2 scale on the right; *d*, summer insolation at 65° N (ref. 59); *e*, $\delta^{18}\text{O}$ in the V 19-30 deep-sea core⁶⁸ for *Uvigerina Senticosa* with an indication of the successive climatic stages.

According to CLIMAP members⁴⁵, the last interglacial was similar to the current climate. However, they noted some indications of a slightly warmer climate. The Vostok record brings quantifiable evidence of atmospheric warming over Antarctica during this period. This is useful information because there is some controversy about the behaviour of the East Antarctic ice sheet during the last interglacial⁴⁵. In addition, the Vostok record does not support an interglacial character for marine stage 5c (first part of E), an argument used to suggest an East Antarctic ice surge around 95 kyr BP (ref. 51).

Whereas glacial buildups in the deep-sea record (Fig. 4e) are gradual, giving the ice-volume cycles their asymmetrical saw-toothed character⁵², the ice record (Fig. 4b) shows that the minimum temperatures were of the same order during cold periods (H, F, D and B stages). This may be linked to the rapid response of the atmospheric system to forcings and feedbacks and also suggests that there is a very stable mode of the climatic system for these cold periods. Such differences in shape were previously noted between ice-volume and temperature signals recorded in some oceanic cores⁵³.

Forcing and feedback factors

Beyond this reconstruction of the East Antarctic climate, we now discuss some current important aspects of the mechanisms which have probably controlled this climate and how the East Antarctic climate is related to the global climate. We will examine the possible role of changes in insolation, atmospheric CO_2 content and global ice volume.

Insolation changes. Over recent years, the Milankovitch hypothesis that orbital variations are the principal causes of the Pleistocene ice ages has received considerable support^{45,52-54}, and has stimulated many modelling efforts⁵⁵⁻⁵⁸. The spectral analysis of proxy records has revealed periodicities statistically correlated to the precessional (19 and 23 kyr), obliquity (41 kyr) and eccentricity (100 kyr) cycles. There is a general consensus

that summer insolation at about 65° N might be a plausible forcing as Northern Hemisphere ice sheets have undergone the major changes, and that the dominant 100-kyr cycle would result from a nonlinear response of ice sheets to orbital forcing.

Before discussing the possible link between the Northern Hemisphere insolation changes and the Vostok climate, we will investigate whether there is a relationship between this climate and the local changes of insolation.

The comparison of the $\delta^{18}\text{O}$ ice profile (Fig. 4b) with the annual insolation curve for 80° S (ref. 59) (Fig. 4a) reveals a very striking relationship between the cold and warm isotope stages and the minima and maxima of the insolation curve. While discontinuous sampling prevents us from performing reliable spectral analysis, especially for short periodicities, this remarkable correlation strongly supports the role of orbital forcing in climatic change.

The particular importance of the obliquity cycle, previously suggested from some high-latitude oceanic cores⁵⁴, would indicate that orbital forcing acts at a local scale. At 80° S, the maximum change of annual insolation is ~7% (ref. 59) which, from the radiative equilibrium equation, corresponds to a direct temperature effect of 4 °C. This direct effect is certainly important when compared with the 10 °C temperature range inferred from the $\delta^{18}\text{O}$ profile.

The apparent climatic synchronicity between the Northern and Southern Hemispheres poses a problem because the seasonality history of the polar insolation at 65° is different for the two hemispheres^{60,61}. One explanation⁶¹ could be that, for polar regions, the climate responds to annual insolation changes that are in phase for the two hemispheres. The correlation between the 80° S annual insolation and the $\delta^{18}\text{O}$ record gives some support to this hypothesis.

Finally, accepting the role of orbital forcing on the Southern Hemisphere climate, the existence of a roughly 40-kyr cycle in the $\delta^{18}\text{O}$ ice core record indirectly supports the Vostok core dating.

Atmospheric CO_2 concentration. CO_2 measurements from the analysis of air bubbles entrapped in ice from Greenland and Antarctic cores have shown that CO_2 changes are associated with climatic changes. Such direct measurements are available for the past 40 kyr (refs 62-65) and show a CO_2 increase of ~40% between the last glacial maximum and the pre-industrial Holocene period. These measurements suggest that CO_2 and its greenhouse warming might be involved in the control of the ice ages²¹ and also in their interhemispheric synchronicity^{42,60}.

Shackleton *et al.*⁶⁶ have used the idea that the difference in carbon isotope ratios in surface and deep oceanic waters varies with CO_2 levels⁶⁷ to develop an indirect approach which extends the atmospheric CO_2 record further back in time. They estimate this parameter from the $\delta^{13}\text{C}$ difference between planktonic and benthic foraminifera ($\Delta\delta^{13}\text{C}$). The validity of this approach is supported by the fact that the estimated CO_2 values are consistent with the polar record over their common part⁶⁶ (the past 40 kyr). However, because many assumptions underly this method, it has to be corroborated either from other oceanic cores or, more directly, from ice cores. A study is in progress to obtain a detailed CO_2 profile along the Vostok core to examine directly the CO_2 -climate connection over the past 150 kyr. In the following discussion, we will treat the $\Delta\delta^{13}\text{C}$ curve as a CO_2 curve.

The first 150 kyr of this $\Delta\delta^{13}\text{C}$ record⁶⁸ is shown on Fig. 4c. One can easily see the similarities between this curve and the $\delta^{18}\text{O}$ Vostok record. A relationship between CO_2 content and atmospheric temperatures is expected. Indeed, climate models predict that the greenhouse CO_2 effect should be amplified in high latitudes especially near ice margins^{69,70}. Thus, these observed similarities suggest a CO_2 -atmospheric temperature connection all over the last climatic cycle. According to Stauffer *et al.*²¹ a 40% CO_2 change corresponds to a temperature rise of 2-4.5 °C in high latitudes. Thus, the hypothesis that CO_2 changes are an important factor in controlling high latitude climate is well within the realm of possibility.

There are several other features of the CO₂-climate relationship. CO₂ measurements at Dome C indicate that the increase in CO₂ concentration associated with the end of the last ice age began before, or simultaneously with, the climatic change at high southern latitudes⁶⁵. On the other hand, from the 340-kyr reconstructed CO₂ curve, Pisias and Shackleton⁷¹ inferred that CO₂ lags the orbital forcing (summer insolation at 65° N) but leads the ice volume, by an average of 2.5 kyr. From this, they made two important points^{68,71}: (1) CO₂ changes are not a consequence of climatic changes but may represent part of the mechanisms by which orbital variations induce changes in climate; (2) the presence of a strong obliquity component suggests a high-latitude control for the mechanisms by which CO₂ responds to orbital forcing.

This also puts constraints on the way in which CO₂ levels in the ocean surface, which eventually control the atmospheric CO₂ levels, can vary⁶⁸. Recently, four groups⁷²⁻⁷⁵ have independently explored CO₂ scenarios implying a high latitude control through: the changes in ocean circulation; the influence of the amount of light reaching the polar regions on the utilization of nutrients; and a combination of these two factors.

The Knox and McElroy nutrient scenario⁷⁴ includes a mechanism whereby small changes in insolation at high latitudes could result in large changes in CO₂ and thus looks attractive in view of the possible CO₂ climatic role at the timescale considered here although this type of scenario⁷⁸ is not supported by recent results on planktonic foraminifera from Antarctic deep-sea sediments.

Ice-volume feedbacks. Although many assumptions are involved, the above discussion suggests that local insolation and possibly the atmospheric CO₂ have played a part in determining the Antarctic climate over the past 150 kyr. The two factors alone, however, would not explain all the climatic variability seen in the $\delta^{18}\text{O}$ Vostok record. In particular, we observe that the very cold stage F corresponds to an intermediate inferred CO₂ value.

Note that this stage F corresponds to the time of lowest insolation at 65° N (Fig. 4d)⁵⁹. (Indeed, there are similarities between this insolation curve and the $\delta^{18}\text{O}$ Vostok record (Fig. 4b) over all the past 150 kyr.) Modelling of the growth and retreat of the ice sheets has shown that ice volume changes can be driven by summer radiation in Northern Hemisphere high latitudes^{56,57,76,77}. Although all of the models do not specifically refer to 65° N, the generated ice-volume curves resemble, with a few kiloyears lag, the summer insolation at this latitudinal band. As shown in Fig. 4d, high volumes correspond to low insulations and vice versa. Such generated ice volume curves differ from ice volumes deduced from benthic foraminifera (Fig. 4e). In particular, note the difference during stage F. According to Budd⁶¹ the two approaches of deriving ice volumes can be reconciled if the contribution of ^{18}O -poor Antarctic ice is taken into account. This raises the question of how Northern Hemisphere ice extent can influence Antarctic climate.

Although we cannot answer this question here, we note that recent GCM results⁴² show that such a relationship cannot be explained by the interhemispheric exchange of heat in the atmosphere. Other mechanisms would have to be involved, such as a change in the cross-equatorial heat transport by the ocean circulation^{42,60,78} or a response of the East Antarctic ice sheets to sea-level changes⁶¹. This second hypothesis is valid only if its apparent contradiction with the time lead of Antarctic sea-ice compared with the Northern Hemisphere ice volume can be solved as suggested by Budd⁶¹.

Conclusions

We have obtained the first complete and unambiguous isotope description from a deep-ice core of the last climatic cycle. The 2,083-m Vostok record covers the past 150 kyr extending into the end of the previous glacial period. The timescale has been established by combining a glaciological model appropriate here as the core represents only about the first half of the ice sheet, with an original approach for estimating past accumulation rates from the isotope record. While this method has its own limita-

tions, it has the advantage of being independent of other palaeo-indicators or climatic forcing series.

This $\delta^{18}\text{O}$ record has been interpreted as essentially representing temperature changes over East Antarctica. This interpretation is based on the $\delta^{18}\text{O}$ -temperature gradient observed for modern precipitation and on a dynamically-simple isotope model. Although aware of the necessity of a more detailed modelling of the atmospheric $\delta^{18}\text{O}$ cycle using GCMs, we have confidence in the temperature interpretation as far as major $\delta^{18}\text{O}$ changes are concerned.

In this interpretation, the Vostok record provides extremely rich climatic information. The present Holocene period was preceded by a long glacial period marked by two relatively warm interstadials. The well-marked last interglacial was significantly warmer than the Holocene and the end of the previous glacial was quite similar to the last glacial maximum.

A comparison with $\delta^{18}\text{O}$ in deep-sea cores shows that the Antarctic climate and the global ice volume have not evolved in the same way. The last interglacial appears to be about twice as long in the Antarctic temperature record as in the ice-volume record. In addition, there are marked differences in the shapes of the records during the last glacial. Thus, we now have access to another different, but complementary, climatic record fully covering the last climatic cycle.

The presence of a roughly 40-kyr cycle in the Vostok record strongly supports the role of orbital forcing in determining climatic changes. This record is approximately in phase with the annual insolation at 80° S suggesting that this forcing operates at a local scale. There are also some common features between the climatic curve and the summer insolation at 65° N which may be the result of the influence of the Northern Hemisphere ice sheets on the Antarctic climate.

There are striking similarities between the $\Delta\delta^{13}\text{C}$ curve of Shackleton and co-workers^{66,68} and the $\delta^{18}\text{O}$ record. Following these authors in treating the $\Delta\delta^{13}\text{C}$ curve as a CO₂ curve, there appears to be, over a complete climatic cycle, a relationship between atmospheric CO₂ levels and atmospheric temperatures as previously suggested for the past 40 kyr.

There are several other ways to obtain more climatic information from the Vostok ice than the $\delta^{18}\text{O}$ method described here. Interesting results concerning ^{10}Be have been obtained²⁷, and other studies are either in progress or planned for the near future. They include additional deuterium and oxygen-18 determinations and measurements of total gas volume, crystal size, CO₂ in the air bubbles, $^{18}\text{O}/^{16}\text{O}$ ratio of oxygen in air and aerosol content.

The Vostok ice core offers the possibility of measuring many other parameters and their variation with time. As shown from other ice cores, the number of these parameters of interest is growing. Recent studies include more and more chemical elements⁷⁹⁻⁸³ and trace gases⁸⁴ as well as $^{12}\text{C}/^{13}\text{C}$ ratio in CO₂ (ref. 85). Although their first objective is to reconstruct the Earth's palaeo-environment, some of these parameters also contain, at least indirectly, palaeoclimatic information. Finally, it is hoped to reach greater depths in the Vostok drilling and thus to extend the time scale considerably.

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A phage repressor-operator complex at 7 Å resolution

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The crystal structure of a complex between the DNA-binding domain of phage 434 repressor and a synthetic 434 operator shows that the protein, very similar in conformation to λ repressor, binds to B-form DNA with the second α -helix of a helix-turn-helix motif lying in the major groove.

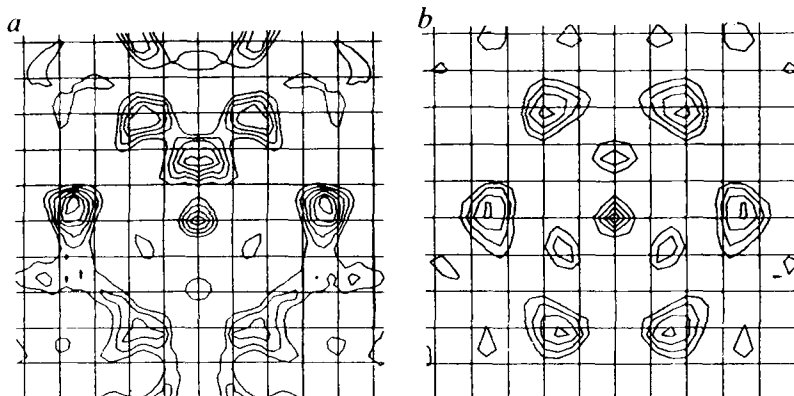
WE present the X-ray crystal structure at 7 Å resolution of a repressor protein bound to its operator DNA. The protein (also a positive regulator of gene expression) is the amino-terminal, DNA-binding domain of the repressor encoded by coliphage 434, a close relative of phage λ . The operator is a 14-base-pair (bp) synthetic oligonucleotide with 2-fold symmetry that is specifically recognized by 434 repressor. Determination of the structure was facilitated by the preparation of isomorphous repressor-DNA crystals using operator DNA with 5-bromodeoxyuridine instead of thymine at two symmetry-related positions. Important aspects of the structure include: (1) The repressor conformation, predominantly α -helical, is remarkably similar to that of the first four α -helices of λ repressor¹. (2) The DNA conformation is B-like, with some deviation at the ends of the operator. (3) Repressor contacts the DNA backbone at several distinct points. Bushman *et al.*² elsewhere in this issue present evidence that these are sites of strong interaction between repressor and DNA phosphates. (4) An α -helix (α 3) lies in the

major groove of the DNA and another α -helix (α 2) lies across the groove. The helices α 2 and α 3 correspond to the helix-turn-helix motif found in the crystal structures of three other DNA-binding proteins^{1,3,4} (and postulated to occur in many more, including 434 repressor⁵⁻⁷). The mode of binding to DNA is similar to that proposed for those proteins on the basis of model building^{1,8-10,22}, with the α 3 helix disposed so that its residues can make sequence-specific contacts with DNA. Changing these residues in the α 3 helix of 434 repressor can change the specificity of the repressor (ref. 11 and accompanying paper¹²).

Structure determination

Crystals of the complex of the 434 repressor DNA-binding domain R1-69 and synthetic 434 operator DNA were prepared as described previously¹³. The space group is I422, with $a = b = 166.4$ Å, $c = 139.4$ Å. Each strand of the synthetic DNA operator had the sequence 5'-HO-d(A-C-A-A-T-A-T-A-T-A-T-G-T)-OH 3'. Derivative crystals were also prepared, using a similar

Fig. 1 Noncrystallographic Harker section. *a*, The section of the difference Patterson that lies 45.6 Å from the origin and perpendicular to the body diagonal of the unit cell. The body diagonal intersects the section at its centre. The small squares of the grid are 5 Å on a side. The set of six peaks corresponding to vectors between equivalent Br atoms on adjacent 14mers forms a roughly hexagonal array, with each peak lying ~18 Å from the body diagonal. Peaks from analogous Br atoms on straight B-DNA with its helix axis coincident with the body diagonal would lie 9.5 Å from the centre. *b*, The same section after 3-fold averaging about the body diagonal, using the programs of Bricogne¹⁸.



oligonucleotide with 5-bromodeoxyuridine instead of dT at position 7. (Both 14mers were purchased from Pharmacia P-L Biochemicals.) Data were collected to 7 Å resolution using a Xenonics imaging proportional counter¹⁴ with CuK α radiation from an Elliott GX-6 rotating anode X-ray generator. Integrated intensities were obtained as described by Durbin *et al.*¹⁴ and processed using programs from P. R. Evans (MRC Laboratory of Molecular Biology, Cambridge, UK). Combination statistics are shown in Table 1. The mean isomorphous difference was

Table 1 Data combination statistics

	R_{sym}	N	$\bar{I}$
Native	0.087	1,171	270
Br	0.066	1,134	455

N , number of measurements; $\bar{I}$, mean intensity; $R_{\text{sym}} = \sum_h |I_h - \bar{I}_h| / \sum_h I_h$.

small (0.12 for 1,105 common reflections). The consequent noisy difference Patterson could not be interpreted directly, but the positions of the Br atoms were obtained by a procedure that took advantage of the previously described analysis of packing in these crystals¹³.

Protein-DNA complexes in the 1422 crystals are stacked in rods with noncrystallographic 3₁ symmetry, parallel to the body diagonals of the unit cell. The rise per repeat of the 3₁ axis is 45.6 Å, so that the section of the difference Patterson perpendicular to the body diagonal and intersecting it 45.6 Å from the origin is a 'noncrystallographic Harker section', in the sense that it contains peaks representing vectors between atoms related by the noncrystallographic symmetry. On this section of the Patterson, we therefore expected to find a set of 3-fold-related peaks corresponding to vectors between Br atoms on different 14mers. Moreover, the known chemical structure of the derivative restricted the range of possible distances of these peaks from the noncrystallographic symmetry axis. Figure 1 shows the pattern actually found, before and after 3-fold averaging. It is similar to the pattern expected on the basis of the packing model of Anderson *et al.*¹³, in which the DNA is stacked end-to-end in straight rods parallel to the unit cell body diagonals, but the peaks are ~5 Å farther from the axis than predicted. We found that B-DNA with a slight right-handed supertwist placed Br atoms in positions that matched the observed pattern much better. The local tilt of the DNA in this model was 12°.

Assignment of the Br-Br vectors on the noncrystallographic Harker section gives coordinates of the Br positions relative to the 3₁ axis, but leaves undetermined one translational degree of freedom, the displacement of this noncrystallographic symmetry axis from the body diagonal of the unit cell. There is also one origin ambiguity (see Fig. 2). The ambiguity was resolved,

and the translational displacement determined, using a search procedure in which calculated Br contributions and centric $|F_{\text{der}} - F_{\text{nat}}|$ were compared (by means of a correlation function) between 15 and 7 Å resolution. The details will be reported elsewhere (J.E.A., J. Moulai and S.C.H., in preparation). A peak in the correlation function gave a trial solution, which was refined by conventional methods¹⁵ (Table 2).

Native phases were determined and used to compute an initial electron-density map, which was inspected on an Evans and Sutherland PS300 interactive graphics system using the program FRODO (ref. 16). The map was noisy and contained no easily recognizable features, but averaging with respect to the noncrystallographic 3₁ axis gave some indication of a double-helical DNA backbone and several rod-like features where protein was expected.

Phases were refined by exploiting the redundancy of information present in the noncrystallographic 3₁ symmetry^{17,18}. In drawing the envelope required for phase refinement^{17,18}, it proved helpful to use a model map that included the N-terminal domain of λ repressor¹ and DNA as a guide. The model consisted of the coordinates of λ -repressor residues 17-85 (ref. 1) (to match the size of R1-69), positioned to fit a straight double helix of DNA aligned along the noncrystallographic 3₁, and, instead of straight DNA, the coordinates of the supertwisted

Table 2 Br atom position refinement

Resolution (Å)	N	m	$f_{\text{rms}}/E_{\text{rms}}$	R_K
13.12-11.67	143	0.46	1.74	0.045
11.67-10.50	189	0.42	1.58	0.061
10.50-9.55	260	0.45	1.75	0.059
9.55-8.75	306	0.42	1.56	0.058
8.75-8.08	362	0.43	1.58	0.078
8.08-7.50	377	0.39	1.53	0.082
7.50-7.00	407	0.38	1.40	0.132
13.12-7.00	2,028	0.42	1.55	0.075

Comparison of initial and refined Br atom positions

Site	Initial	Refined	Occupancy
1	(0.16, -0.20, -0.01)	(0.161, -0.195, -0.013)	0.903
2	(-0.03, -0.26, 0.19)	(-0.033, -0.259, 0.190)	1.001
3	(-0.02, -0.29, 0.23)	(-0.020, -0.289, 0.227)	0.788
4	(-0.21, -0.48, 0.27)	(-0.211, -0.480, 0.270)	0.781
5	(-0.24, -0.47, 0.31)	(-0.242, -0.467, 0.307)	0.960
6	(-0.31, -0.66, 0.51)	(-0.306, -0.661, 0.511)	0.905

Statistics were calculated after 10 cycles of phasing and refinement¹⁵, using 1422 data expanded to space group I222, starting with the positions calculated from model B-DNA with a right-handed supertwist ($\alpha = -12^\circ$; ref. 21) of the C-7 atoms of the thymine in position 7 of the 14mer sequence. m , Figure of merit; f_{rms} , root-mean-square heavy atom structure factor; E_{rms} , root mean square lack of closure error; $R_K = \sum |F_{\text{PH}}^{\text{obs}} - F_{\text{PH}}^{\text{calc}}| / \sum |F_{\text{PH}}^{\text{obs}}|$.

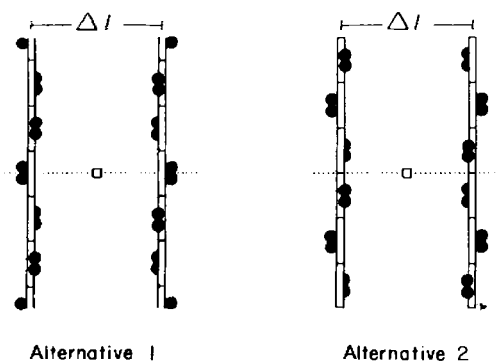


Fig. 2 Schematic projections down the 4-fold axis c (squares) of two of the four rods most closely associated with a lattice point, showing the two possible choices of origin (alternatives 1 and 2). The translational displacement, Δl (one of the parameters searched as described in the text) is shown for each. In alternative 1, the dyad between two Br atoms (filled circles) is coincident with the diagonal crystallographic dyad [110] (dotted line). In alternative 2, the dyad between two operators (rectangles) is coincident with the crystallographic dyad.

DNA used to locate the Br atom positions, with its superhelical axis coincident with the noncrystallographic symmetry axis. Inspection of the electron density computed from 7 Å structure factors of this model helped us to recognize features of the averaged initial map around which to draw the envelope. A total of six cycles of phase refinement were performed. The statistics are shown in Table 3 and part of the final map in Fig. 3. The positions of Br atoms are shown as stippled spheres in Fig. 3. Ribbons of density corresponding to double-helical DNA backbone are clearly visible, and it is easy to distinguish major and minor grooves. Rod-like density features (clearly low-resolution α -helices) are also visible next to the DNA on the opposite side from the Br atoms. One of these lies in the major groove of the DNA, and another is roughly at right angles to it. Their size and relative orientation show that they correspond to the helix-turn-helix motif seen in λ repressor¹, λ cro³ and catabolite gene activator protein⁴, and their position on the operator is consistent with modes of binding suggested for those proteins by model building^{1,8-10,22}.

Structure of R1-69

A backbone model for λ repressor¹ can be superimposed on the electron-density map of the co-crystal in such a way that $\alpha 2$ and $\alpha 3$ (the helix-turn-helix motif) are well aligned in rod-like density features (Fig. 4). Inspection shows that two other 'sausages' of density correspond closely to the $\alpha 1$ and $\alpha 4$ helices of λ repressor. The axis of the $\alpha 1$ density is displaced from the axis of the λ -repressor $\alpha 1$ helix by 2–3 Å, and its orientation is slightly different. The N-terminus of 434 repressor $\alpha 1$ density is close to the C-terminus of $\alpha 3$ density, and com-

parison with the λ repressor model suggests that it corresponds to the N-terminus of the polypeptide chain. The fit of the λ -repressor $\alpha 4$ helix to the co-crystal density is even closer than that of $\alpha 1$, although not quite as good as $\alpha 2$ and $\alpha 3$. The final helix of the λ -repressor domain ($\alpha 5$, not shown in Fig. 4) does not lie in clearly rod-like density, suggesting that the fold of the 434 repressor polypeptide chain differs significantly from the λ -repressor fold in this region. The λ -repressor helix $\alpha 5$ mediates a close dimer contact between N-terminal domains. Such a close contact may indeed not occur in 434 repressor, because in the absence of DNA, R1-69 crystallizes as a monomer. (The space group of the R1-69 crystals is $P2_12_12_1$, with one protein molecule per asymmetric unit; S. Almo, J.E.A. and S.C.H., unpublished data.) The links between helices are not very clear in the co-crystal map (only the $\alpha 2$ and $\alpha 3$ 'sausages' are bridged by density at the level we chose to contour), but the correspondence between the λ -repressor model and rod-like density features makes us confident that we have correctly determined connectivity and chain direction. We conclude that R1-69 has a conformation very similar to that of the DNA-binding domain of λ repressor. It contains at least four α -helices, and their orientation and packing are similar in the two structures. The 434 repressor lacks the N-terminal arm of λ repressor, and the C-terminal portion of the domain may follow a different course.

Operator structure

Only the DNA backbone is clearly delineated at this resolution; density corresponding to base pairs is less regular. Ribbons of density follow fairly closely the path of the backbone of 10.5 bp per turn of B-DNA (Fig. 3), with a deviation of 2–3 Å towards the 3' ends of each strand which is described below. The positions of the Br atoms indicate that the local axis of the DNA is displaced by a few ångströms from the noncrystallographic 3_1 axis. In fitting the Br positions, we used a smooth supercoil with precise stacking of base pairs at the ends of adjacent 14mers, but the map suggests that a better fit can be obtained with a straight B-DNA model for each 14mer, tilted at $\sim 12^\circ$ with respect to the 3_1 and displaced from it by 4–5 Å, so that the register of the double helix is not maintained from 14mer to 14mer. This model produces nearly perfect overlap of the purine rings of the 5' adenine bases from successive 14mers; the 3' thymines are left unstacked. This exposure may in part account for the deviation of the 3' end of the backbone density from the B-DNA model, and a contact with an R1-69 monomer bound to the adjacent 14mer may also be involved (see below). A perturbation of strict B-DNA structure to fit the density involves a displacement of the 3' end of the 14-base strand by 2–3 Å (Fig. 5). As the 5' end of the opposite strand is not correspondingly displaced, some distortion of normal base-pair geometry may be required to accommodate the backbone shift. The shift seems significant only for the four 3'-terminal bases.

Protein-DNA contacts

As shown in Figs 3, 4 and 5, R1-69 is bound to DNA with $\alpha 3$ inserted in the major groove, a feature anticipated by previous models of repressor-DNA interaction^{1,3,4,8-10,22}. The axis of $\alpha 3$ is displaced by ~ 2 Å from the centre of the major groove. The position of the helix with respect to the base pairs seems to be determined by a set of contacts that the repressor domain makes with the DNA sugar-phosphate backbone (Fig. 5). One of these contacts is near the N-terminus of $\alpha 4$, where a strong bridge connects protein and DNA densities near the position of the phosphate 5' to base +10. (For the numbering of phosphate groups, see Fig. 5.) A less striking bridge occurs near the adjacent phosphate, 5' to base +9. Another strong bridge joins density from the N-terminus of $\alpha 2$ to density from the DNA backbone of the adjacent 14mer, near the phosphate between the two bases at the 3' end of the strand. In phosphate ethylation interference experiments using the 14mer sequence embedded in continuous DNA, Bushman *et al.*² infer that corresponding phosphates are contacted by intact repressor in solution. Ethylation

Table 3 Noncrystallographic symmetry phase refinement¹⁸

Cycle	R	r	Mean phase change
1	0.493	0.436	62.4°
2	0.397	0.618	17.2°
3	0.361	0.688	9.6°
4	0.340	0.732	7.4°
5	0.330	0.751	5.6°
6	0.324	0.761	3.9°
Overall phase change			68.9°

$$R = \sum |F_{\text{obs}} - F_{\text{calc}}| / \sum |F_{\text{obs}}|; \text{ mean phase change} = \sum |\phi_{\text{calc}} - \phi_{\text{obs}}| / N;$$

$$r = \frac{\sum |F_{\text{obs}}| |F_{\text{calc}}| - \sum |F_{\text{obs}}| \sum |F_{\text{calc}}|}{[\sum |F_{\text{obs}}|^2 - (\sum |F_{\text{obs}}|)^2][\sum |F_{\text{calc}}|^2 - (\sum |F_{\text{calc}}|)^2]}.$$

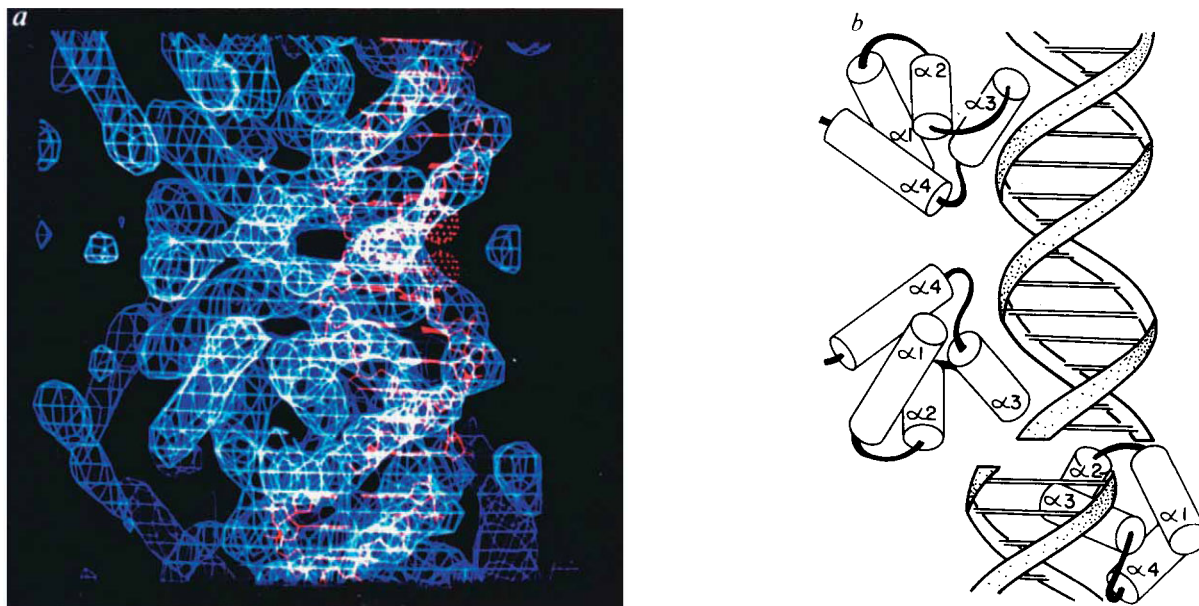


Fig. 3 *a*, Overview of the co-crystal map at 7 Å resolution after six cycles of noncrystallographic symmetry averaging and phase refinement. Almost an entire 1422 asymmetric unit, consisting of three R1-69 monomers and one-and-a-half 14mers, is shown. Each protein-DNA complex consists of two protein monomers and one operator. The view is perpendicular both to the noncrystallographic 3_1 and to the dyad of a complex. The positions of Br atoms are shown as stippled spheres. Model B-DNA, tilted and displaced as described in the text, is superimposed on DNA electron density. *b*, Schematic representation of *a*, with α -helices (cylinders) labelled near their N-termini.

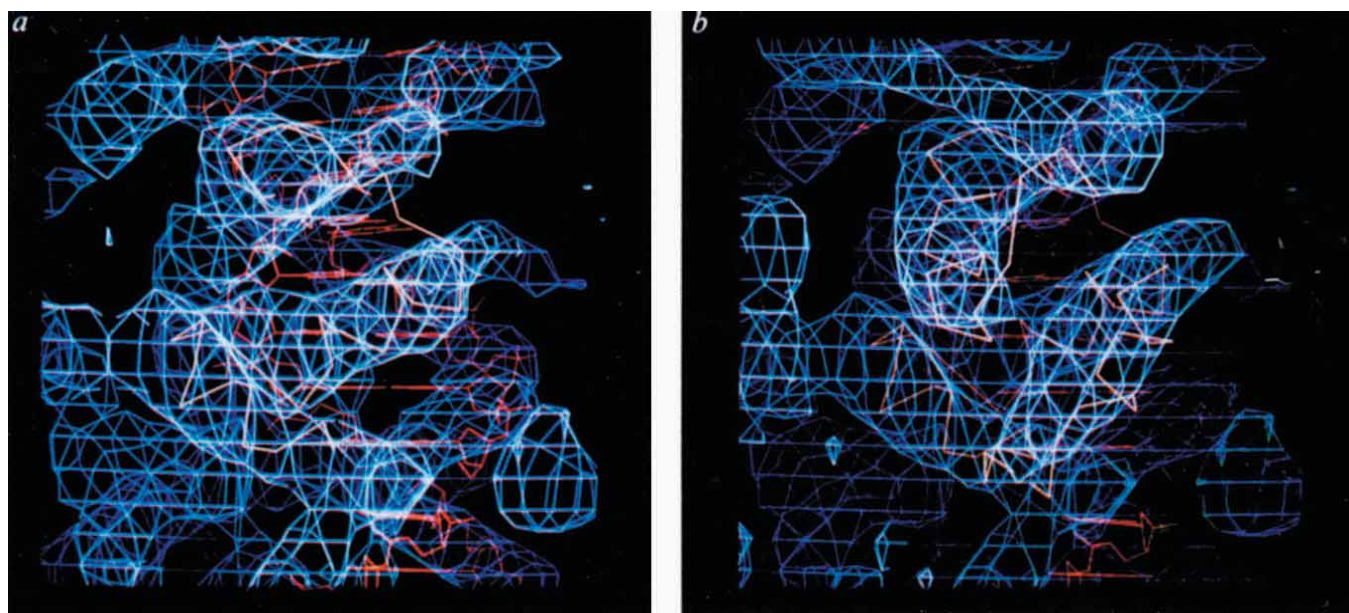
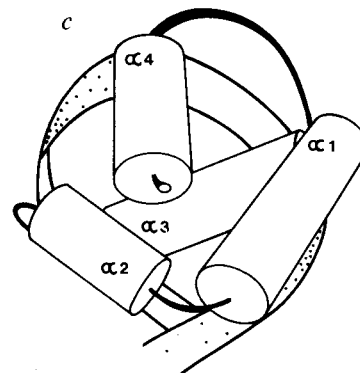


Fig. 4 Structure of R1-69. Straight B-DNA (red) and the α -carbon backbone of λ -repressor residues 17-75 (ref. 1) (gold), including the C-terminal half of $\alpha 1$ and all of $\alpha 2$ - $\alpha 4$, are superimposed on the electron density (see text). *a*, View perpendicular to the noncrystallographic symmetry axis of part of a monomer of R1-69 (the lower one of the dimer in Fig. 3). Helix $\alpha 3$ lies in the major groove of the DNA and $\alpha 2$ lies across $\alpha 3$. A strong bridge of density connects the N-terminus of $\alpha 2$ to the DNA backbone near phosphate +14 (see Fig. 5*b* for phosphate numbering) of the adjacent 14mer. At the top, bridges between protein and DNA are partially visible near phosphates +9 and +10. *b*, View in the same orientation as *a*, but farther from the DNA, showing helices $\alpha 1$ (lower right) and $\alpha 4$ (upper left). Helices $\alpha 2$ and $\alpha 3$ are visible in the background. *c*, Schematic representation of *b*, with the α -helices (cylinders) labelled near their N-termini.



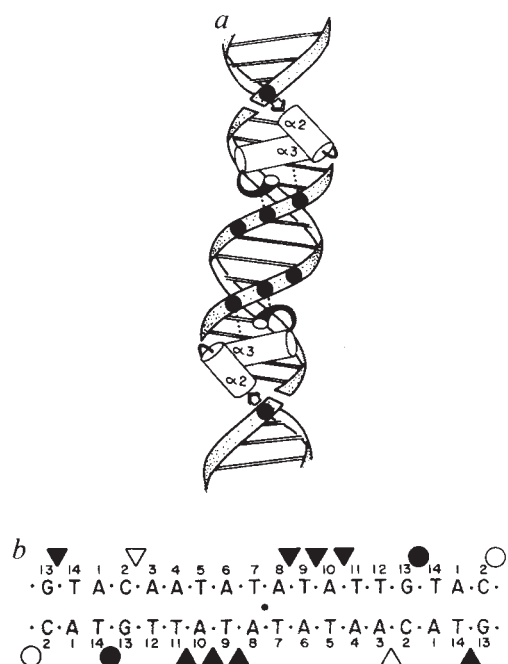


Fig. 5 Protein-DNA backbone contacts. *a*, Schematic drawing showing the contacts. The helix-turn-helix structures and part of the protein backbone just C-terminal to $\alpha 3$ are shown for a dimer of R1-69. The diagram includes one entire 14mer and the ends of the adjacent 14mers, drawn as straight B-DNA. The tilt of the adjacent 14mers is slightly exaggerated. Filled circles indicate positions where the locations of contacts between protein and DNA phosphates suggested by the crystal structure and those inferred from ethylation interference experiments² agree. Dotted lines connect the phosphates to the regions of the protein believed to provide the contacts. The part of the protein responsible for the phosphate contact nearest the centre of the operator is not known. The arrows show the directions of the displacements seen in the crystal of the 3' ends of the DNA strands of the adjacent 14mers (see text). These displacements result in part from contacts made by the N-termini of the $\alpha 2$ helices of the dimer bound to the central 14mer. The corresponding displacements of the 3' ends of the strands of the central 14mer, resulting from proteins bound to the adjacent 14mers, are not shown. *b*, The DNA sequence corresponding to *a*, showing the positions of possible contact between protein and DNA suggested by the crystal structure, and giving the phosphate numbering scheme used in the text. Bases belonging to the same 14mer are connected by small dots. The large dot in the centre locates the 2-fold axis of the central 14mer. Triangles, contacts made by the R1-69 dimer associated with the central DNA 14mer; circles, contacts made by proteins bound to the flanking 14mers. Filled symbols have the same meaning as in *a*; open symbols indicate positions where a contact is suggested by the crystal structure but not detected by ethylation interference².

of the phosphates 5' to bases -1 (the first base outside the 14mer sequence), +9 or +10 on either strand interferes strongly with repressor binding. We believe that the -1 phosphate contact corresponds to the contact in the crystal between $\alpha 2$ and the adjacent 14mer, and that the other two correspond to the bridges of density in the centre of the operator. These results show that the overall configuration of the complex of intact repressor with DNA is similar to that of R1-69, although it is possible that the details of the interactions are slightly different because of constraints imposed by stable dimerization of the intact molecule.

Strictly speaking, confluences of protein and DNA densities such as those just described imply nothing more than close proximity, but it is reasonable to suppose that a bond will form

if the appropriate type of residue is nearby. Groups most likely to interact directly with negatively charged DNA phosphates are those that are positively charged and those that can donate hydrogen bonds. Our confidence that we have correctly identified helices and their connectivity, and how they relate to the amino-acid sequence, permits us to enumerate amino-acid residues that could be involved in these interactions. We suggest that Asn 16 or Gln 17 (or both) might participate in the contact at the N-terminus of $\alpha 2$, Lys 40, Arg 41, or Arg 43 could be involved in the +9 and +10 contacts; some residue C-terminal to $\alpha 4$ might also contact the +9 phosphate. We also note that helices $\alpha 2$, $\alpha 3$ and $\alpha 4$ are arranged so that the N-termini of all three point towards DNA phosphates. The N-terminus of an α -helix carries a virtual partial positive charge¹⁹ which could produce favourable interactions with DNA phosphates to stabilize binding in the observed orientation. A role for interactions between the N-terminal ends of α -helices and backbone phosphates in the binding of λ repressor to DNA has been suggested previously¹.

With the relationship between protein and DNA in our interpretation of the electron density, it is not possible for Asn 16 or Gln 17 to hydrogen bond to the -1 phosphate if the operator is embedded in continuous, straight DNA. If the continuous DNA is given a slight supertwist, however, then both residues are brought to within reasonable hydrogen-bonding distance of the phosphate². This suggests that if the ethylation interference at the -1 phosphate involves disruption of hydrogen bonds donated by Asn 16 or Gln 17, then the DNA is slightly bent (to a radius of curvature of ~ 100 Å) by repressor binding. A slight bend would also enhance any charge interaction that exists between the N-terminus of $\alpha 2$ (see above) and the -1 phosphate. Other deformations of the double helix are possible, but super-twisting (or bending) is the simplest and most easily interpretable. The displacement of 14mers from the noncrystallographic 3₁ axis, as well as the displacement of the 3' end of the DNA strand seen in the crystal (see above and Fig. 5*a*), may be related to this suggested tendency of repressor to bend DNA when it binds². A monomer of R1-69 bound to a particular 14mer in the crystal can make the $\alpha 2$ contact (which we propose is made to the -1 phosphate on bent continuous DNA in solution) only by lateral displacement of the neighbouring 14mer. The consequently unstacked 3' end of this neighbour might then adjust further to optimize contact with the N-terminus of $\alpha 2$.

There are two other interactions between protein and DNA backbone suggested by our interpretation of the electron density. The N-terminus of $\alpha 3$ closely approaches the DNA backbone, placing the side chain of Ser 30 in a position to hydrogen bond to the phosphate 5' to base +11. Ethylation at that phosphate decreases binding, although less strongly than the modifications described above². Finally, just beyond the C-terminus of $\alpha 3$, an arm of density reaches towards the opposite DNA strand near the phosphate 5' to base +3. The location and orientation of the arm suggest a salt bridge between that phosphate and the side chain of Lys 38, but ethylation at that position has little effect on binding².

Side chains on the external surface of $\alpha 3$ (those that would be exposed to the solvent if not in the complex) are clearly in position to make sequence-specific contacts to the bases in the major groove. The excellent fit of the λ -repressor backbone and of B-DNA to features of the electron density, the invariance in three other structures of the helix-turn-helix motif^{5,20}, and the assignment by sequence similarity of corresponding residues in the λ and 434 repressors^{6,7} permit limited model building of specific interactions between protein and DNA by insertion of 434 repressor side chains onto λ -repressor backbone positioned as in Fig. 5. An important preliminary conclusion is that base pairs 1-4 seem to be accessible to direct interaction with repressor side chains on the $\alpha 3$ helix, with contacts possible between Gln 28 and base pair 1, Gln 29 and base pairs 2 and 3, and Gln 33 and base pairs 3 and 4. In addition, residues in the turn between $\alpha 3$ and $\alpha 4$ can probably contact base pair 5

and possibly base pair 6. We are attempting to test these suggestions and are extending the resolution of the structure determination to 3.2 Å.

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Changing the binding specificity of a repressor by redesigning an α -helix

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We replaced amino acids on the 'outside', or solvent-exposed, surface of the DNA recognition α -helix of 434 repressor with the corresponding amino acids from the recognition helix of P22 repressor. The binding specificity of the resulting hybrid protein, as measured in vivo and in vitro, was that of P22 repressor.

THE X-ray crystal structure of the bacteriophage 434 repressor bound to its DNA operator is described in the accompanying paper¹. One striking feature of the structure is that an α -helix of the protein ($\alpha 3$, or the recognition helix) lies in the major groove of the B-form operator DNA where it presumably makes sequence-specific contacts. This helix is part of a conserved 'helix-turn-helix' unit found in the structures of three other sequence-specific DNA-binding proteins (λ repressor, λ cro protein, and *Escherichia coli* catabolite gene activator protein (CAP))²⁻⁶. For each of these proteins, model building has suggested that the recognition helix can be placed in the major groove of the relevant operator⁷⁻⁹. The conserved helix-turn-helix unit has been predicted to exist in many other DNA-binding proteins based on amino-acid sequence homologies¹⁰⁻¹². Analyses of mutant proteins¹³⁻¹⁸ are consistent with the proposal that at least part of the specificity of binding is determined by the recognition helix in each of several cases.

In the experiments described here, we have redesigned the recognition helix of 434 repressor so that the resulting protein has a new biological activity. On the outside (solvent-exposed) surface of the α -helix, the redesigned 434 repressor bears amino acids that are presumed to lie at the corresponding positions on the recognition helix of a different repressor, that encoded by the *Salmonella* bacteriophage P22. Amino acids on the 'inside' surface of the helix were unchanged. We reasoned that this strategy would allow the new amino-acid sequence to fold into an α -helix in the context of the remainder of the protein. *In vivo*, the parental 434 repressor binds only to 434 operators, but the redesigned 434 hybrid repressor, named 434R [$\alpha 3$ (P22R)], binds only to P22 operators. *In vitro*, purified 434R [$\alpha 3$ (P22R)] binds specifically to P22 operator DNA. Furthermore, it binds to the three binding sites in wild-type P22 O_R with the same affinity order as does P22 repressor, namely $O_{R1} \approx O_{R2} > O_{R3}$. The apparent affinities of P22 repressor and 434R [$\alpha 3$ (P22R)] for P22 operator DNA are comparable. We conclude first, that in 434 repressor, amino acids along the recognition helix are solely responsible for distinguishing between binding to two different sets of operators; and second, 434 repressor and P22

repressor present their recognition helices in very similar ways to 434 operators and P22 operators, respectively.

Redesigning the recognition helix

The presumptive configurations of the recognition helices and flanking residues of the 434 and P22 repressors are shown in Fig. 1a. The arguments that led to the assignment of these particular configurations are summarized in Fig. 1 legend. To redesign 434 repressor, we assumed that residues on the inside surface of its helix, which pack against the body of the repressor, could not be substituted without destabilizing the protein. We also assumed that amino acids on the outside surface, which are free to interact with DNA in the protein-DNA complex, could be substituted without strongly affecting the stability of the protein. Figure 1b shows the presumptive configuration of the redesigned recognition helix and flanking residues of 434R [$\alpha 3$ (P22R)]. Compared with wild-type 434 repressor, 434R [$\alpha 3$ (P22R)] bears five amino-acid substitutions taken from P22 repressor.

Figure 1c shows the sequence of a fragment of the gene encoding 434R [$\alpha 3$ (P22R)], and, schematically, the strategy by which it was constructed. Using mismatched primer mutagenesis *in vitro*, we introduced restriction enzyme cleavage sites that bracket the region encoding the recognition helix in the 434 repressor gene. This region was then removed from the 434 repressor gene by cleavage with the appropriate restriction enzymes, and replaced with a new DNA sequence, constructed from four synthetic oligonucleotides, which encodes the hybrid helix of 434R [$\alpha 3$ (P22R)]. The hybrid gene was fused to the *lac* promoter to create the plasmid pRW219. When pRW219 was introduced into an appropriate *E. coli* strain, ~1% of cellular protein was 434R [$\alpha 3$ (P22R)] (data not shown).

434R [$\alpha 3$ (P22R)] activity *in vivo*

E. coli cells that are producing the repressor encoded by a particular phage are immune to infection by that phage. Because P22 is a *Salmonella* phage which does not grow in *E. coli* strains, hybrid phages named λimm^{434} and λimm^{P22} were used in the

Fig. 1 Redesigning the outside surface of the recognition helix of 434 repressor. *a*, Schematic representations of the presumptive recognition helices of the 434 and P22 repressors. *b*, Two schematic representations of the presumptive configuration of the recognition helix of 434R [$\alpha 3$ (P22R)]. *c*, Construction of the gene encoding 434R [$\alpha 3$ (P22R)]. *a*, The dispositions of amino acids were assigned by superimposing the conserved helix-turn-helix units predicted to exist in the 434 and P22 repressors¹⁰ on the helix-turn-helix unit seen in the X-ray crystal structure of λ repressor². We have previously presented results that support this disposition of amino acids in the recognition helix of 434 repressor¹³, and Anderson *et al.* have recently demonstrated the existence in 434 repressor of a helix-turn-helix unit. The Gly residue, present at the turn in almost all predicted helix-turn-helix units¹⁵, is triply underlined for reference. The amino-acid sequence shown for P22 repressor is actually that of the *pcA* mutant P22 repressor³⁰, in which Lys replaces the Glu of wild-type P22 repressor at residue 42. Residue 42 is the underlined lysine in the following sequence in the figure: ... Ser-Lys-Thr-Glu ... No differences between the DNA-binding activities of wild-type P22 repressor and *pcA* mutant P22 repressor have been observed *in vivo*³⁰ or in an extensive series of measurements *in vitro* (J. Douhan III and M.P., unpublished data; see also Fig. 3). Therefore, in constructing the gene encoding 434R [$\alpha 3$ (P22R)], we chose not to change the residue at the corresponding position in 434 repressor, Lys 38. *b*, Amino acids taken from P22 repressor are drawn in white letters inside black boxes. The dashed lines at either end of the first drawing indicate that this helix is embedded in the remainder of the 434 repressor protein. The second representation³¹, a view from the carboxy-terminal end of the helix in projection down the helical axis, emphasizes the spatial segregation of P22 repressor-derived amino acids on the outside surface of the helix (white letters in black boxes) and 434 repressor-derived amino acids on the inside surface of the helix (black letters). Note that the amino-acid substitution at position -1 of the recognition helix is not present in this second representation and that the recognition helices of 434 repressor and P22 repressor have common amino acids at positions 4, 6 and 8. *c*, A portion of the gene encoding 434R [$\alpha 3$ (P22R)] is shown with the corresponding amino-acid sequence. The conserved glycine residue is again triply underlined for reference (see *a*). The five amino-acid substitutions in 434R [$\alpha 3$ (P22R)] are marked by arrows. DNA to the left of the *KpnI* site and to the right of the *DdeI* site encodes the 193 amino acids that are identical in 434 repressor and 434R [$\alpha 3$ (P22R)]. DNA between the *KpnI* and *DdeI* sites was assembled from four synthetic oligonucleotides which are represented by two white and two stippled boxes. Also shown are sites for the restriction enzymes *MboI* and *TaqI* which were used in the experiments described in Fig. 4. These sites are common to the genes encoding 434 repressor and 434R [$\alpha 3$ (P22R)].

Methods. A site for the enzyme *KpnI* was introduced into the gene encoding 434 repressor to create the plasmid pRW199. We used an oligonucleotide with the sequence: 5'-TGCTGGGTGGTACCCACCTT-3' which was mismatched with bases in the wild-type gene at the two underlined nucleotides. Mutagenesis was performed as described previously¹³ using the methods of Zoller and Smith³² with the following exceptions: single-stranded DNA was prepared from the *dam*⁻ *E. coli* strain RW102 (our unpublished data), and the template DNA was a derivative of the plasmid, pEMBL8+ (ref. 33). The four synthetic oligonucleotides shown in *c* which encode part of 434R [$\alpha 3$ (P22R)] were assembled essentially as described by Sgarbetta and Khorana³⁴, and the resulting 39-bp duplex which had *KpnI*- and *DdeI*-compatible ends was isolated by gel electrophoresis. This DNA fragment was substituted for the corresponding 39-bp fragment of pRW199 essentially as described previously¹³.

Table 1 Immunity specificity of 434R [$\alpha 3$ (P22R)] *in vivo*

Protein	Immunity to	
	λ imm ⁴³⁴	λ imm ^{P22}
434 R	+	-
P22 R	-	+
434 R [$\alpha 3$ (P22 R)]	-	+

Phages were spotted lawns of three host strains, each of which was producing one of the repressors listed in the column on the left. Following overnight incubation, the lawns were examined for evidence of plaque formation and host killing. + Indicates that host cells were immune to infection (no apparent cell lysis) and - indicates the converse. Identical results were obtained following overnight growth at either 30 or 37 °C. λ imm⁴³⁴ and λ imm^{P22} are hybrid phages in which the repressor gene and operators of phage λ are replaced by the repressor gene and operators of phages 434 and P22, respectively^{19,20}. These phages behave with regard to their immunity properties exactly as do the parental phages, 434 and P22^{19,20}. In all cases where phage growth occurred, phage spots were turbid except when λ imm⁴³⁴ was spotted on cells producing 434R [$\alpha 3$ (P22R)]. Each of the three proteins in the table was at least 1% of cellular protein. The bacterial host strains for these experiments were derivatives of XA90 (ref. 26) which carried an F'(lacI^{Q1}) episome²⁷ as well as the plasmids listed below. Spot tests were performed in the presence of 1 mM isopropyl β -D-thiogalactopyranoside (IPTG) to induce the synthesis of each of the repressors whose genes are transcribed by *lac* operon promoters. Listed below for each repressor is the name of the plasmid that was introduced into XA90 cells to direct synthesis of the protein, and the estimate of the percentage of total protein that consisted of repressor in these cells based on electrophoresis in SDS-polyacrylamide gels²⁸ of total cellular protein: 434 repressor, pRW88 (ref. 13), 1% (or pRW190, 10%, our unpublished data); P22 repressor, pTP15 (ref. 29), 1%; 434R [$\alpha 3$ (P22R)], pRW 219 (see Fig. 1), 1%.

experiments described below. In these hybrid phages, the repressor gene and operators of phage λ have been replaced by the repressor gene and operators of phages 434 and P22, respectively^{19,20}. *In vivo*, the DNA-binding activity of 434 repressor or P22 repressor is extremely specific: cells producing 434 repressor are immune to infection by λ imm⁴³⁴ phages but not immune to infection by λ imm^{P22} phages¹⁹, whereas cells producing P22 repressor are immune to infection only by λ imm^{P22} phages²⁰. This binding specificity is maintained even when cells are producing very large quantities of either 434 or P22 repressor (see Table 1). (For reference, Fig. 2 shows consensus DNA sequences for 434 and P22 operators.)

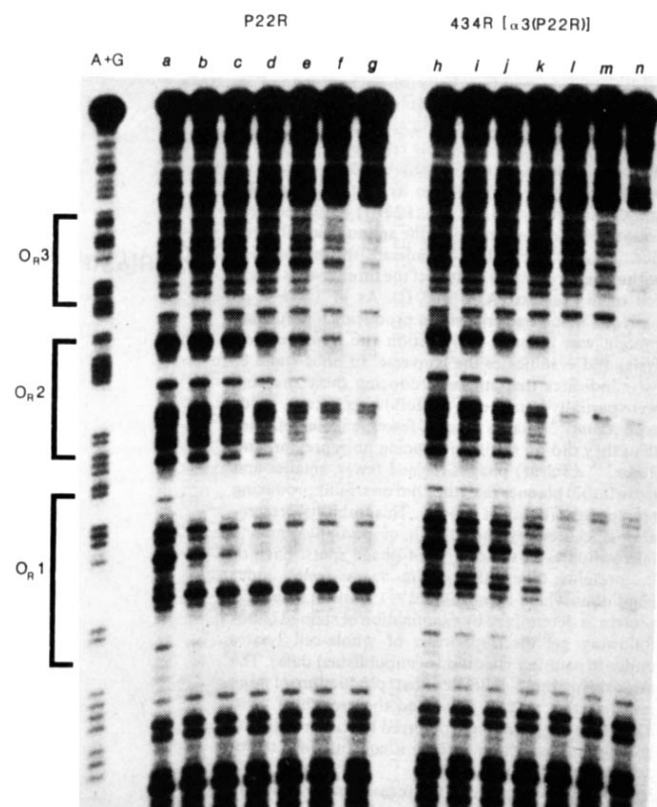
To test the DNA-binding specificity of the hybrid repressor, 434R [$\alpha 3$ (P22R)], we spotted λ imm⁴³⁴ phages and λ imm^{P22} phages on a lawn of cells producing 434R [$\alpha 3$ (P22R)]. These cells were immune to infection by the λ imm^{P22} phages and not immune to infection by the λ imm⁴³⁴ phages (Table 1). The degree of immunity conferred was indistinguishable from that conferred by wild-type P22 repressor: when 10⁸ λ imm^{P22} phages were spotted on a lawn of cells producing 434R [$\alpha 3$ (P22R)],

A . T . A A G C T T . A . T P22 Operator
A C A A T T G T 434 Operator

Fig. 2 Consensus sequences for P22 and 434 operators. Shown are consensus sequences along one DNA strand for P22 and 434 operators^{13,23}, aligned at their centres of symmetry which are indicated by ●. An × in either sequence indicates a non-conserved base at that position of the operator.

Fig. 3 Binding of 434R [$\alpha 3$ (P22R)] to P22 O_R *in vitro*. Autoradiogram resulting from a typical DNase I protection experiment in which purified P22 repressor or 434R [$\alpha 3$ (P22R)] was bound to DNA containing wild-type P22 O_R . End-labelled DNA fragments in the lane marked A+G were cleaved at purines to generate size markers. The boundaries of each 18-bp binding site (O_{R1} , O_{R2} , and O_{R3}) are indicated on the left of the figure. Lanes a-g represent the electrophoresis of binding reactions that contained successively higher concentrations of P22 repressor. Lanes h-n represent the electrophoresis of binding reactions that contained successively higher concentrations of 434R [$\alpha 3$ (P22R)]. In all the reactions, the concentration of O_R -containing DNA fragments was $\sim 10^{-10}$ M. The concentrations of active protein monomers in each binding reaction were as follows: a, 0; b, 7.6×10^{-10} M; c, 1.3×10^{-9} M; d, 2.2×10^{-9} M; e, 3.7×10^{-9} M; f, 6.2×10^{-9} M; g, 1.1×10^{-8} M P22 repressor; and h, 0; i, 5.2×10^{-9} M; j, 8.8×10^{-9} M; k, 1.5×10^{-8} M; l, 2.5×10^{-8} M; m, 7.0×10^{-8} M; n, 5.6×10^{-7} M 434R [$\alpha 3$ (P22R)]. At saturating concentrations of each protein, both P22 repressor and 434R [$\alpha 3$ (P22R)] protect from DNase I cleavage regions of equivalent size, even though the precise boundary of the protected region below O_{R1} is not readily apparent because DNase I cleaves only weakly in this region. Examination of over-exposed autoradiograms reveals that the region protected by both proteins extends to the G residue 5 bp outside O_{R1} on the labelled strand. We note that a single phosphodiester bond within O_{R1} is not protected from DNase I cleavage by binding of P22 repressor although it is protected by 434R [$\alpha 3$ (P22R)]. (This phenomenon has been observed previously²⁴.) Using a DNA fragment labelled on the other strand, we observed that all of the phosphodiester bonds within O_{R1} were protected by each protein. The protein used in the experiment shown in the figure was *pcA* P22 repressor (see Fig. 1 legend). We have observed no significant differences between the binding of wild-type P22 repressor and *pcA* P22 repressor to O_R DNA. A detailed analysis of the binding of wild-type P22 repressor to O_R has been reported by Poteete and Ptashne²⁴. In the experiment shown here, we observed half-maximal occupancy of O_{R1} at a concentration of 1.0×10^{-9} M P22 repressor and at 8.8×10^{-9} M 434R [$\alpha 3$ (P22R)]. In nitrocellulose filter-binding experiments³⁵, the concentrations of 434R [$\alpha 3$ (P22R)] and P22 repressor monomers required to retain on the filter one-half of the DNA fragments in binding reactions were 1.6×10^{-10} M and 3.5×10^{-9} M, respectively (data not shown). In these experiments, the concentration of DNA fragments was 5×10^{-12} M, and the binding reaction conditions were slightly different (see methods). The concentrations of P22 *pcA* repressor required for half-maximal operator occupancy that we have determined in these experiments are virtually identical to those determined earlier for wild-type P22 repressor^{22,24}. From DNase I protection experiments similar to that shown here, we deduced the following ratios of protein concentrations required for half-maximal occupancy of O_{R1} , O_{R2} and O_{R3} on a DNA fragment-bearing wild-type O_R : for P22 *pcA* repressor, 1:1.5:6; and for 434R [$\alpha 3$ (P22R)], 1:1:6. The affinity order of P22 *pcA* repressor is identical to that determined earlier for wild-type P22 repressor²⁴. In the absence of cooperative interactions, threefold higher concentrations of P22 *pcA* repressor or of 434R [$\alpha 3$ (P22R)] were required to occupy O_{R3} half-maximally than were required to occupy O_{R1} half-maximally in DNase I protection experiments in which each site was present on a separate DNA fragment (data not shown). For each of three proteins, we list below the highest concentration tested at which we did not detect specific binding to non-cognate operator DNA and the ratio of this value over the concentration at which specific binding to cognate operator DNA was detectable: 434 repressor, 4×10^{-6} M, 100; P22 *pcA* repressor, 2×10^{-7} M, 300; 434R [$\alpha 3$ (P22R)], 1×10^{-6} M, 100.

Methods. 434R [$\alpha 3$ (P22R)] was purified from cells containing pRW219 following the same protocol by which 434 repressor has been purified³⁶. We measured the stability of 434R [$\alpha 3$ (P22R)] by digesting it with the thermo-stable protease thermolysin at various temperatures¹⁴. The amino-terminal domain of wild-type 434 repressor was stable to digestion at temperatures as high as 60°C whereas the amino-terminal domain of 434R [$\alpha 3$ (P22R)] was stable to digestion only at temperatures as high as 37°C (data not shown). We have not investigated further this apparent instability of 434R [$\alpha 3$ (P22R)] *in vitro*. Three different end-labelled DNA fragments were used in the binding experiments described in the figure: a *HindIII*/*CfoI* fragment from pTP37 (ref. 24) which contains wild-type P22 O_R ; an *EcoRI*/*HindIII* fragment from pRW253 which contains O_{R1} ; and an *EcoRI*/*HindIII* fragment from pRW254 which contains O_{R3} . (The asterisk indicates the labelled end of each fragment.) pRW253 and pRW254 were generated by partially cleaving a fragment of pTP37 with *HinfI* (which cleaves within O_{R2}), and then separately cloning the appropriate O_{R1} - and O_{R3} -containing DNA fragments into pEMBL8+. The reaction conditions for DNase I protection experiments were: 10 mM HEPES (pH 7.0), 200 mM KCl, 1 mM MgCl₂, 0.5 mM CaCl₂, 100 μ g ml⁻¹ sonicated chick blood DNA at 23°C. The reaction conditions for the nitrocellulose filter binding experiment described in the legend were identical except that the divalent cations and chick blood DNA were omitted.



no lysis of the cells was apparent. Also, production of 434R [$\alpha 3$ (P22R)] had no detectable effect on the number or size of plaques formed by λ imm⁴³⁴ phages (see Table 1). Thus, the five amino-acid substitutions introduced into 434 repressor to create 434R [$\alpha 3$ (P22R)] have switched the biological specificity of the protein from 434 operator binding to P22 operator binding.

Another observation from these physiological tests supports our conclusion that 434R [$\alpha 3$ (P22R)] has a negligible affinity for 434 operators. Wild-type λ imm⁴³⁴ phages (encoding a functional repressor) form turbid plaques when spotted on lawns of either wild-type cells or cells producing P22 repressor, whereas mutant λ imm⁴³⁴ phages (encoding a defective repressor) form clear plaques on the same lawns. We found that wild-type λ imm⁴³⁴ phages, when spotted on a lawn of cells producing 434R [$\alpha 3$ (P22R)], formed clear rather than turbid plaques (see Table 1). We imagine that this interference with the function of the repressor encoded by the infecting λ imm⁴³⁴ phages is a result of the fact that heterodimers of 434 repressor and 434R [$\alpha 3$ (P22R)] do not bind to 434 operators. We know that 434 repressor, which binds as a dimer, is a two-domain protein in which the amino-terminal domain mediates DNA binding

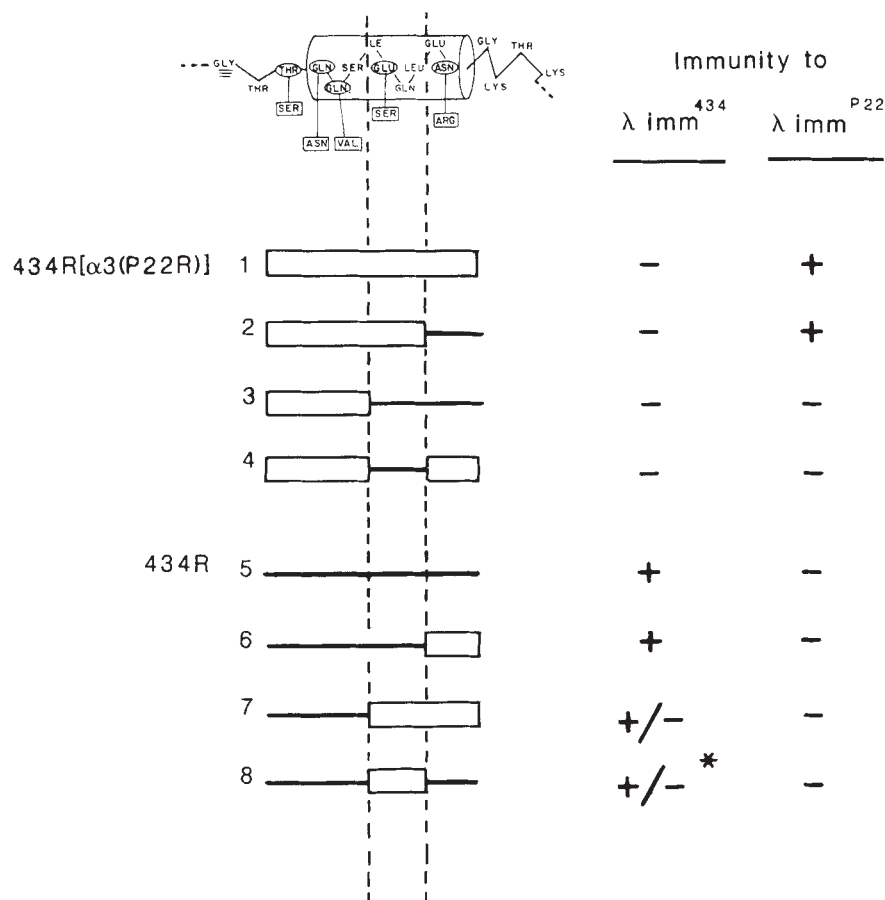
and the carboxy-terminal domain mediates dimerization^{13,21}. As 434R [$\alpha 3$ (P22R)] and wild-type 434 repressor have the same carboxy-terminal domains, they presumably form heterodimers efficiently. In the presence of excess 434R [$\alpha 3$ (P22R)], virtually all of the 434 repressor in cells infected with wild-type λ imm⁴³⁴ phages would be sequestered in the form of mixed dimers; these mixed dimers evidently do not bind with significant affinity to 434 operator DNA.

434R [$\alpha 3$ (P22R)] activity *in vitro*

Purified 434R [$\alpha 3$ (P22R)] bound specifically to DNA containing the right operator (O_R) of phage P22 in DNase I protection experiments (Fig. 3). P22 repressor and 434R [$\alpha 3$ (P22R)] identically protected the same regions of O_R -containing DNA from DNase I cleavage with one exception: P22 repressor did not protect a single phosphodiester bond within O_{R1} that is protected by binding of 434R [$\alpha 3$ (P22R)] (see Fig. 3). As expected, based on the operator specificity exhibited by 434R [$\alpha 3$ (P22R)] *in vivo* (see Table 1), we failed to observe specific binding of 434R [$\alpha 3$ (P22R)] (or P22 repressor) to 434 operator DNA even at high repressor concentrations (see Fig. 3 legend). Similarly, we failed to observe specific binding of 434

Fig. 4 Activity *in vivo* of recombinants between 434 repressor and 434R [$\alpha 3$ (P22R)]. The diagram on the left indicates schematically the identity of the amino acids in the recognition helix of each protein. P22 repressor-derived amino acids from 434R [$\alpha 3$ (P22R)] are indicated by open boxes and 434 repressor-derived amino acids by solid lines. For reference, we show a drawing of the recognition helix of 434 repressor in which each of the five amino acids that were substituted in creating 434R [$\alpha 3$ (P22R)] is circled and connected to a box containing the amino-acid substitution. Dashed vertical lines indicate the boundaries within the recognition helices of the three groups referred to in the text (A, B and C). As in Table 1, + indicates that a bacterial strain producing a particular protein was immune to infection (no apparent cell lysis) and - indicates the converse. In lines 7 and 8, +/- indicates that strains producing these proteins were partially immune by the following criteria: wild-type λ imm⁴³⁴ phages formed fewer, smaller plaques than they did on strains producing no repressor, and λ imm⁴³⁴ c (clear) phages formed fewer, smaller and more turbid plaques than they did on strains producing no repressor (data not shown). This turbidity presumably is caused by the growth of partially immune microcolonies in the centres of phage spots. Each of the proteins, except that of line 8, was tested under conditions where it constituted ~1% of total cellular protein as determined by examination of stained bands following gel electrophoresis of whole-cell lysates under denaturing conditions (unpublished data). The asterisk in line 8 indicates that production of high levels of this protein prevented the growth of cells. Therefore, the immunity conferred by this protein was tested under conditions where it constituted <1% of cellular protein.

Methods. Genes encoding the recombinants shown in lines 3 and 7 were generated by crossing the genes encoding 434 repressor and 434R [$\alpha 3$ (P22R)] at the *Mbo*I restriction enzyme cleavage sites common to the DNA fragments encoding their recognition helices (see Fig. 1c). Then the genes encoding the recombinants shown in lines 2, 4, 6 and 8 were generated by crossing the appropriate combinations of genes encoding the proteins shown in lines 1, 3, 5 and 7 at the *Taq*I restriction enzyme cleavage sites common to the DNA fragments encoding their recognition helices. In the DNA sequence shown in Fig. 1c, another site for the enzyme *Taq*I exists, but cleavage at this site is blocked by methylation *in vivo* at the overlapping site for the enzyme *Mbo*I. All the recombinant genes were ligated into a common backbone from the plasmid pRW220, and the resulting plasmids were then transformed into XA90(F' *lac*^{Q1}) cells for immunity tests (see Table 1). pRW220 is a derivative of pRW88 (ref. 13) in which the 248-bp *Eco*RI/*Hind*III fragment was replaced by the ~75-bp *Eco*RI/*Hind*III fragment from the plasmid π VX (ref. 37). The DNA sequence of the relevant portion of each of the genes was confirmed by the chain termination method³⁸. The level of each protein *in vivo* was estimated as described in Table 1.



repressor to P22 operator DNA *in vitro*. Comparable concentrations of either P22 repressor or 434R [$\alpha 3$ (P22R)] monomers were required to occupy O_R DNA half-maximally in several different DNA-binding experiments (see Fig. 3 legend). We cannot assign precise values for the affinity of the dimer of either protein as we do not know the value of the equilibrium constant for the dimerization reaction of either protein. Dimerization of P22 repressor and of 434 repressor (and therefore presumably also of 434R [$\alpha 3$ (P22R)]) is mediated in each case mainly by association of carboxy-terminal domains^{21,22}; these domains are different in the two proteins.

The experiment shown in Fig. 3 also demonstrates that a more subtle characteristic of the binding of 434R [$\alpha 3$ (P22R)] to P22 operators was similar to that of P22 repressor. In O_R , there are three similar but not identical 18-base-pair (bp) sites (named O_{R1} , O_{R2} and O_{R3}) (ref. 23), and P22 repressor binds to these sites on a DNA molecule bearing wild-type O_R with the affinity order $O_{R1} \approx O_{R2} > O_{R3}$ (ref. 24). As shown in Fig. 3, 434R [$\alpha 3$ (P22R)] bound with the same affinity order as did P22 repressor. Both P22 repressor and 434 repressor (and therefore presumably also 434R [$\alpha 3$ (P22R)]) bind cooperatively to adjacent operator sites on a single DNA molecule, and this cooperativity is mediated by the carboxy-terminal domain of each protein^{13,21,22,24}. We also measured the relative affinities of each protein for O_{R1} and O_{R3} in the absence of cooperative interactions. In DNase I protection experiments with O_{R1} and O_{R3} on two different DNA molecules, P22 repressor and 434R [$\alpha 3$ (P22R)] each bound with higher affinity to O_{R1} than to O_{R3} (see Fig. 3 legend).

Subdividing helix 3

We wished to determine whether all of the five amino-acid substitutions introduced in creating 434R [$\alpha 3$ (P22R)] were required for the ability of the protein to bind to P22 operators. Therefore, we took advantage of restriction enzyme cleavage sites present inside the regions encoding the recognition helices of 434 repressor and 434R [$\alpha 3$ (P22R)] (see Fig. 1c). We divided the five amino-acid substitutions into three groups (referred to below as groups A, B and C) and studied the activity *in vivo* of the various recombinants shown in Fig. 4. Group A consists of the three amino-acid substitutions at positions -1, 1 and 2 in the recognition helix; group B consists of the single amino-acid substitution at position 5; and group C consists of the single amino-acid substitution at position 9. All of the proteins referred to in Fig. 4 were stable *in vivo* by the following criterion: when the *lac* promoter driving expression of each protein was fully derepressed, each protein constituted ~1% of cellular protein (data not shown).

We find that, first, the P22 repressor-derived amino acids of groups A and B were necessary and sufficient to determine P22-operator binding specificity. Because we have not attempted to separate the three amino acids in group A, we cannot specify the minimum number of amino-acid substitutions required for P22 operator-binding activity. Second, the 434 repressor-derived amino acids of group A were sufficient for detectable binding to 434 operators, but for full activity (comparable to that of wild-type 434 repressor), the 434 repressor-derived amino acids of both groups A and B were necessary. Third, for all of the

proteins in Fig. 4, the identity of the amino acid in group C (either Asn or Arg) had no detectable effect on the binding activity. Fourth, none of the proteins simultaneously recognized 434 operators and P22 operators.

We also observed that cells producing a 434 repressor protein bearing a single amino-acid substitution, the P22 repressor-derived serine of group B, exhibited the following peculiar property: fewer than 1 in 10^6 cells survived to form colonies when the *lac* promoter driving expression of this protein was fully derepressed. We speculate that the extreme toxicity of this mutant protein may be caused by a drastically enhanced affinity for nonspecific DNA. This phenotype has been observed by Nelson and Sauer²⁵ for a mutant λ repressor that bears a substitution of Ser for Gly at position 5 of its recognition helix.

Conclusions

The experiments reported here extend our earlier work in which we studied a hybrid 434 repressor bearing the recognition helix of 434 *cro* protein¹³. 434 repressor and *cro* protein have very similar DNA-binding activities: they each bind with high affinity to the same set of 434 operators. In addition, the amino-acid sequences of the two proteins are very similar in their DNA-binding domains¹⁰, and it was therefore possible to exchange directly the recognition helix of 434 *cro* protein for that of 434 repressor without otherwise altering either protein. The resulting hybrid repressor was stable and active, presumably because the amino-acid residues on the inside surfaces of the recognition helices of both proteins are nearly identical¹³.

In the experiments reported here, we studied a 434 repressor hybrid that bears the recognition helix of a more distantly related protein. 434 repressor and P22 repressor have different DNA-binding activities. Even at very high concentrations, neither protein binds detectably to non-cognate operators either *in vivo* or *in vitro*. The amino-acid sequences of the two proteins are sufficiently dissimilar that it seemed unlikely that simply substituting the P22 repressor helix for that of 434 repressor would have yielded a stable and functional protein. We therefore engineered the 434 repressor recognition helix, replacing amino acids on its outside surface with the appropriate amino acids of P22 repressor, and leaving amino acids on its inside surface

as 434 repressor amino acids to preserve the structure of the folded protein. The resulting protein bound specifically and with high affinity to P22 operators *in vivo* and *in vitro*. We conclude that the recognition helix of 434 repressor is the sole determinant in 434 repressor that distinguishes between two different specificities: binding to 434 operators or to P22 operators. Some part of P22 repressor other than its recognition helix may also make sequence-specific DNA contacts, but our results require that 434R [$\alpha 3$ (P22R)] make these contacts as well.

As the properties of P22 repressor and 434R [$\alpha 3$ (P22R)] are so similar, we further conclude that the recognition helices of 434 repressor and P22 repressor interact with their respective operators in very similar ways. This conclusion is contrary to other proposals for the recognition helices of λ repressor⁷, λ *cro* protein⁸ and *E. coli* CAP⁹. Model-building studies of possible structures of the protein-DNA complexes formed by these three proteins suggest that each of the three recognition helices interacts with its operator in a significantly different manner.

Our results are consistent with what has been learned from studying the structure of a 434 repressor-operator complex. Anderson *et al.*¹ have observed that α -helix 3 of 434 repressor (its recognition helix) lies in the major groove with its amino-terminal end in intimate contact with the surface of the operator. Our analysis of recombinants between 434 repressor and 434R [$\alpha 3$ (P22R)] suggests that the amino-terminal end of the recognition helix is more important in determining the binding specificity of the protein than is the carboxy-terminal end. Similar suggestions have been made for λ repressor² and *E. coli* CAP⁹ based on model building, and a recent report on the effects of amino-acid substitutions in the recognition helix of λ *cro* protein is also consistent with this idea¹⁸. Further experiments will be required to understand in detail the roles of individual amino acids on the outside surface of the recognition helix in operator binding.

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Reconstitution of an active surface T3/T-cell antigen receptor by DNA transfer

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We have introduced a full-length complementary DNA of the T-cell antigen receptor β -chain into a mutant human T-cell line that lacked a complete β -chain messenger RNA, had a diminished level of α -chain transcript and did not express surface T3- or antigen receptor. Expression of the transfected β -chain led to a normal level of α -chain transcript and a structurally and functionally active T3 T-cell antigen receptor complex on the cell surface.

THE T-cell antigen receptor that functions to recognize foreign antigens is a disulphide-linked heterodimer of relative molecular mass (M_r) 80,000–90,000 (80–90 K), composed of an acidic α - and a basic β -chain^{1–3}. The human^{4–6} and murine^{7–9} cDNAs have recently been cloned, shown to undergo somatic rearrangements in T cells^{4–9} and are related^{4–9} to, but distinct^{10–12} from, immunoglobulin genes. The receptor heterodimer is associated on the cell surface with T3, a set of three non-covalently linked peptides of 20–28 K^{13–17}. In an attempt structurally and functionally to separate the antigen receptor and T3, we selected for mutants of the T-cell tumour line Jurkat, which lacked either T3 or the T-cell antigen receptor, using OKT3 or an antiidiotypic antibody (C305). However, all mutants, selected either way, failed to express both these molecules¹⁶ on the surface. We show here that three mutants that do not express a full length β -mRNA have diminished levels of α -chain specific RNA, and do not express surface T3. Transfection of one of the mutants with β -chain complementary DNA induced the expression of normal levels of α -chain mRNA and led to the expression of a functional T3/T-cell antigen receptor complex on the cell surface.

Intracellular T3 mRNA and proteins

The three subunits of T3 consist of a 26–28 K γ -chain and two chains of 20–30 K, one a glycosylated δ -chain and the other a non-glycosylated ϵ -chain^{18–20}. Using a cDNA probe to the T3 δ -chain (pPGBC-9)²¹, we examined seven T3/T-cell antigen receptor-negative mutants of Jurkat derived from four separate mutagenizations¹⁶ for the presence of transcripts. A Northern blot²² obtained with RNA isolated from four of these mutants, the wild-type cell (Jurkat) and HPB-ALL (Fig. 1a), shows that all mutants examined make transcripts for the T3 δ -chain that are indistinguishable in size from the normal T3- δ transcript.

We also examined wild-type Jurkat and the mutants for intracellular T3 subunits by biosynthetic labelling with ³⁵S-methionine and ³⁵S-cysteine. Several hours of continuous labelling, immunoprecipitation and SDS polyacrylamide gel electrophoresis revealed that all mutants had a doublet at 22–23 K precipitated by anti-Leu 4 from the mutant that is indistinguishable from that detected in Jurkat (Fig. 1b). During shorter labelling periods (15 min), the γ -chain of T3 was also detectable with anti-Leu 4 antibody in all the mutants (data not shown). Endoglycosidase F digestion of the Leu 4 immunoprecipitates from all three cell lines resulted in the loss of only the upper band of the 22–23 K doublet and the appearance of two lower M_r bands of 15 and 17 K (data not shown). These results are consistent with the notion that the upper band of the doublet represents the glycosylated T3 δ -chain and the lower band the non-glycosylated T3 ϵ -chain, as described by others^{18,19,23}.

Either α - or β -chain mRNA in mutants

We examined the cells for transcription of the α - and β -chain genes. Figure 2a is a Northern blot of poly(A)-enriched RNA from Jurkat, along with seven T3 T-cell antigen receptor negative mutants, hybridized to a full-length β -chain probe. Jurkat expresses both a 1.3- and a 1.0-kilobase (kb) β -chain transcript^{24–26}; the 1.3-kb transcript of Jurkat represents a full-length completely rearranged gene containing $V_\beta 1D_\beta 1J_\beta 1C_\beta 1$ (V , variable; D , diversity; J , joining; C , constant regions), whereas the 1.0-kb transcript contains only a $J_\beta 2C_\beta 2$ gene segment²⁷. In three of the seven mutants examined, the 1.3-kb transcript was not detected, although the 1.0-kb transcript was always present (Fig. 2a, lanes 2, 4 and 7). In contrast, the other four mutants contained both 1.3- and the 1.0-kb transcripts that could not be distinguished by size from those detected in the wild-type Jurkat cells (Fig. 2a, compare lane 1 with lanes 3, 5, 6 and 8).

Figure 2b shows that Jurkat also contains a 1.5-kb transcript homologous to the α -chain cDNA probe (lane 1). Four mutants seem to contain greatly diminished levels of α -chain transcripts (lanes 2, 3, 4 and 7). Interestingly, three of these mutants lack the 1.3-kb β -chain transcript (lanes 2, 4 and 7). The remaining three mutants seemed to contain normal levels of both α - and β -chain mRNA.

Transfection with β -chain cDNA

We next investigated the effect of transferring cDNA encoding the β -chain into a mutant that does not express the full-length of 1.3-kb mRNA. We used the Friend spleen focus-forming virus (SFFV) long terminal repeat (LTR)^{28,29} as a transcriptional promoter for the 1.3-kb β -chain transcript from YT35 (ref. 4), which has been shown to be identical to that of Jurkat²⁷. The mammalian selectable marker from the plasmid pSV2-neo (ref. 30) provided positive selection of transfectants (Fig. 3). Protoplast fusion was performed as described previously³¹.

Expression in transfected mutants

Of 15 transfectants, 7 expressed abundant levels of both antigen receptor molecules and T3 antigenic epitopes³². An example of the fluorescence histograms obtained with one such transfected cell, PF-2.8, is compared with those obtained with the wild-type (Jurkat) and the recipient mutant J.RT3–T3.5 (Fig. 4). Note that although the densities of T3 and T-cell antigen receptor determinants in PF-2.8 are less than those observed in the wild-type Jurkat, PF-2.8 expressed abundant levels of both T3 and T-cell antigen receptor determinants compared with the T3/T-cell antigen receptor negative mutant, J.RT3–T3.5. In all seven of the positive transfectants, the relationship between the density of T3 and T-cell antigen receptor epitopes was constant, thus

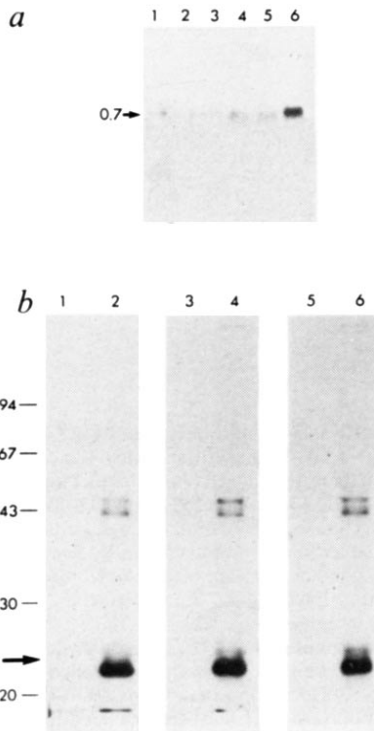


Fig. 1 (Left) *a*, Northern blot analysis of four independently derived T3/T-cell antigen receptor negative mutants of Jurkat hybridized with the T3 δ -chain transcripts. 20 μ g total RNA from the indicated cells were electrophoresed in 1% formaldehyde gels, transferred to nitrocellulose and hybridized with a 32 P-labelled cDNA probe specific for the T3 δ -chain^{21,22}. Lane 1, J.EMS-T3.3; lane 2, Jurkat; lane 3, J.RT3-T3.5; lane 4, J.RT-T3.1; lane 5, S.5; lane 6, HPB-ALL. *b*, SDS-PAGE of biosynthetically labelled T3 antigens in T3 T-cell antigen receptor negative mutants of Jurkat. Lysates of Jurkat (lanes 1 and 2), J.RT-T3.1 (lanes 3 and 4) and J.RT3-T3.5 (lanes 5 and 6) were prepared from cells labelled for 16 h with 35 S-methionine and 35 S-cysteine. Preformed immune complexes prepared with rabbit anti-mouse IgG and either normal mouse serum (lanes 1, 3, 5) or anti-Leu 4 ascitic fluid (lanes 2, 4, 6) were incubated with each lysate and washed according to previously described methods¹⁸. Isolated complexes were then dissolved in SDS sample buffer containing 2-mercaptoethanol and electrophoresed on a 11% polyacrylamide slab gel. Gels were dried and fluorography was performed using sodium salicylate. Shown in the left margin are the relative mobilities of M_r standards run in parallel lanes. The arrow indicates the 22–23 K doublet representing T3 proteins. Note that the higher M_r bands observed between 40 and 50K are probably contaminants as they are also seen in precipitates isolated with normal mouse immunoglobulin preformed complexes.

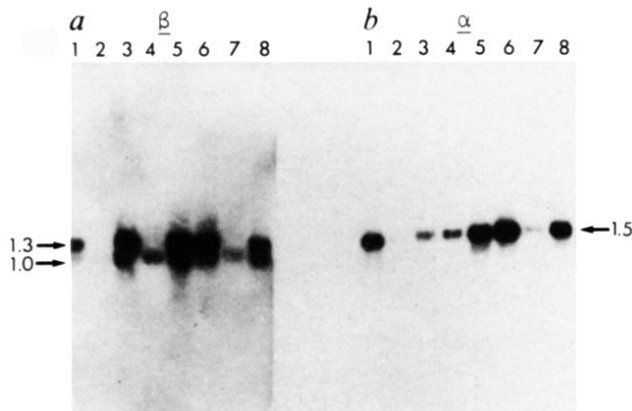


Fig. 2 Northern blot analysis of Jurkat and T3/T-cell antigen receptor negative mutants for β -chain (*a*) or α -chain (*b*) transcripts. 7 μ g poly(A)-enriched RNA were isolated from Jurkat or mutant cells, electrophoresed in a 1% formaldehyde gel, transferred to nitrocellulose paper and hybridized with 32 P-labelled nick-translated probe and washed as described elsewhere²². Probes specific for the β -chain (*a*) or α -chain (*b*) of the antigen receptor heterodimer were previously characterized^{4,6}. Lane 1, Jurkat; lane 2, S.5; lane 3, J.RT-T3.1; lane 4, J.RT-C305.6; lane 5, J.EMS-T3.3; lane 6, J.EMS-C305.1; lane 7, J.RT3-T3.5; lane 8, J.RT3-C305.6. The relative masses (in kb) of the bands obtained are indicated in the margins.

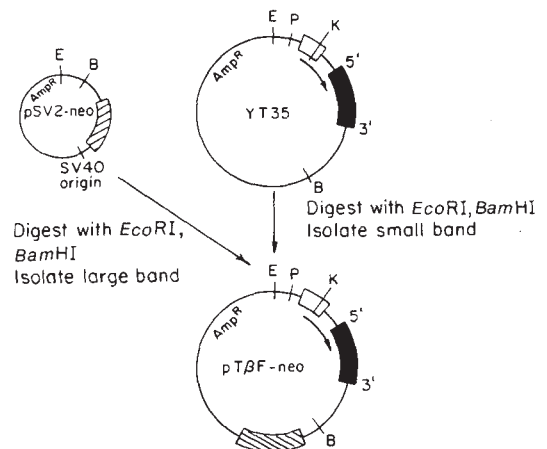


Fig. 3 Construction of plasmid pTBF-neo. The cDNA fragment specific for the β -chain was subcloned into vector pFP502EB511, downstream from the SFFV-LTR, to create plasmid YT35 (ref. 4). pTBF-neo was constructed as illustrated and then transfected into *Escherichia coli* strain DH1. The bacteria were cultured, converted into protoplasts and fused to J.RT3-T3.5 as described in Ochi *et al.*³¹. Cells were plated at a concentration of 10^5 cells per ml in 24-well culture plates and incubated for 48 h. Geneticin was added (2 mg ml^{-1}) to select for transfected clones. □, SFFV-LTR; ■, β -chain cDNA; ▨, neomycin; E, *EcoRI*; B, *BamHI*; P, *PstI*; K, *KpnI*.

reinforcing their interdependence for their surface expression. The remaining eight transfected clones had either minimal or undetectable densities of determinants recognized by OKT3 or C305 monoclonal antibody.

The clone PF-2.8 that expressed the reconstituted T3/T-cell antigen receptor complex was examined by Southern^{33,34} and Northern^{22,35} blot analyses using a nick-translated³⁵ probe to YT35 (ref. 4) to ensure that the new β -chain mRNA was encoded by the transfected DNA, which can be seen at a new 3.3-kb band in the transfected line PF-2.8 (Fig. 5a, lane 3) that is not present in the mutant J.RT3-T3.5 (lane 2) or the wild-type Jurkat cell (lane 1). Several other new bands can also be detected in

PF-2.8, but they probably represent integration events that disrupt the LTR β -chain continuity. These data also show that there are no gross new genomic rearrangements of the β -chain in the mutant J.RT3-T3.5 compared with Jurkat.

Northern blot analysis (Fig. 5b) indicates that two new transcripts homologous with the β -chain are found in PF-2.8 (lane 3). Jurkat, the wild-type cell, contains a 1.3-kb (V-D-J-C rearrangement) and a 1.0-kb (J-C transcript) mRNA (lane 1)²⁷. Lane 2 confirms that the 1.3-kb transcript cannot be detected in J.RT3-T3.5 (ref. 8) and lane 3 shows that the transfected clone expresses new transcripts of 1.9 and 3.4 kb. The increase in size of the β -chain mRNAs presumably results from a

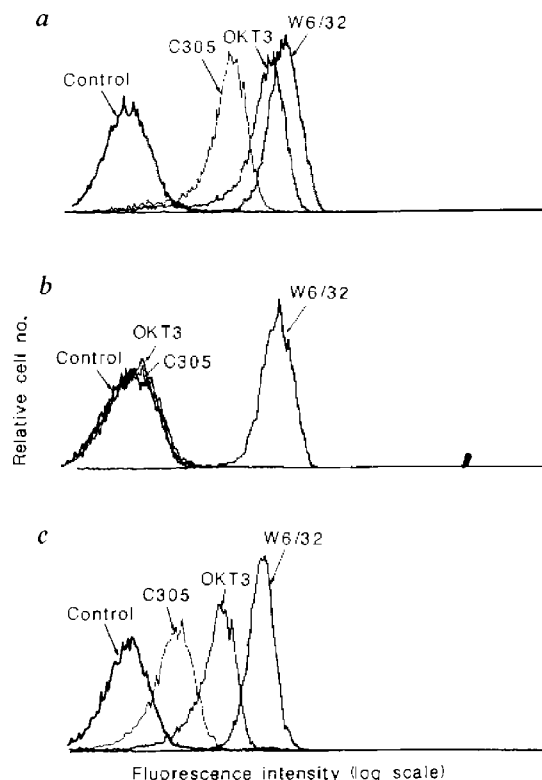


Fig. 4 Immunofluorescence analysis for a T3/T-cell antigen receptor negative mutant of Jurkat transfected with the β -chain cDNA. Initial studies indicated that the SFFV LTR could activate YT35 transcription in the rat cell line Rat-1 (data not shown). Using the technique of protoplast fusion, pTBF-neo was amplified in bacteria and the bacteria fused with the mutant J.RT3-T3.5. This mutant was shown to lack the complete 1.3-kb β -chain transcript and had a decreased level of α -chain mRNA. Transfected cells were selected for geneticin-resistant clones and subsequently expanded in culture. Resistant clones were assayed for surface antigen receptor and T3 with amonoclonal anti-clonotypic antibody, C305 (ref. 16) and monoclonal OKT3 antibody³², respectively, using indirect immunofluorescence as assessed by fluorescence flow cytometry. Jurkat (a); J.RT3-T3.5, a T3 T-cell antigen receptor negative mutant lacking the 1.3-kb β -chain transcript (b); and PF-2.8, a β -chain transfectant of J.RT3-T3.5 (c) were analysed by indirect immunofluorescence using fluoresceinated-Fab/2 goat anti-mouse immunoglobulin on a FACS IV cell sorter. Cells were first stained with a control non-reactive mouse myelome (MOPC 195) or the indicated antibodies: OKT3 (anti-T3), C305 (anti-receptor) or w6/32 (anti-HLA).

combination of different RNA initiation and termination sites of the newly introduced DNA and makes it unlikely that they represent reactivation of the original β -chain gene.

Initial Northern blot analysis (Fig. 2) indicated that the level of α -chain transcripts was diminished in J.RT3-T3.5. Northern blot analysis of RNA of the transfected line revealed that the level of α -chain transcripts in PF-2.8 (Fig. 5c, lane 3) is comparable in magnitude with that observed in Jurkat (lane 1) and clearly increased from the level observed in the mutant J.RT3-T3.5 (lane 2). These results suggest that transfection of the full-length β -chain into J.RT3-T3.5 induces increased levels of α -chain transcripts.

Reconstituted T3/antigen receptor is active

Previous studies have demonstrated that two stimuli are required for the activation of Jurkat, as measured by interleukin-2 (IL-2) production^{36,37}. One stimulus increases cytoplasmic free calcium $[Ca^{2+}]_i$ and can be provided by the lectin phytohaemagglutinin (PHA), calcium ionophores (A23187 or ionomycin) or monoclonal antibodies reactive with the T3/T-cell antigen receptor complex^{37,38}. The second stimulus can be provided by phorbol

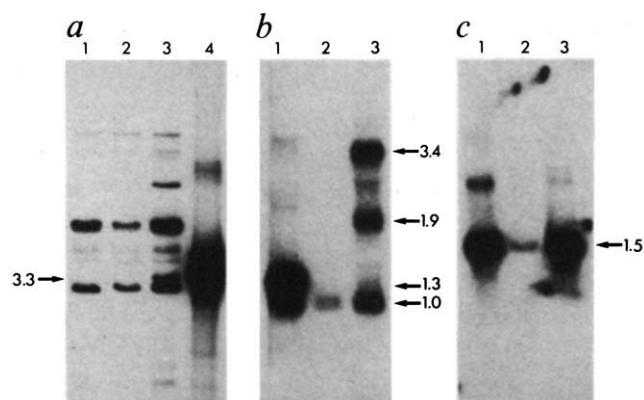


Fig. 5 Molecular analysis of transfectant PF-2.8. a, Southern blot analysis. 10 μ g DNA isolated from Jurkat (lane 1), J.RT3-T3.5 (lane 2), PF-2.8 (lane 3) and pTBF-neo (lane 4) were digested with *Eco*RI and *Bam*HI, electrophoresed in an 0.8% agarose gel, transferred to nitrocellulose and hybridized as described³³. The probe was the ³²P-labelled nick-translated³⁴ *Eco*RI/*Bam*HI fragment of pTBF-neo that contains β -chain cDNA. b, Northern blot analysis for β -chain transcripts in PF-2.8. 7 μ g poly(A)-enriched RNA was isolated from Jurkat (lane 1), J.RT3-T3.5 (lane 2) and PF-2.8 (lane 3) and was electrophoresed in a 1% formaldehyde gel, transferred to nitrocellulose³⁵ and probed with ³²P-labelled nick-translated β -chain cDNA. c, Northern blot analysis for α -chain transcripts in PF-2.8. 7 μ g poly(A)-enriched RNA isolated from Jurkat (lane 1), J.RT3-T3.5 (lane 2) and PF-2.8 (lane 3) were electrophoresed in a 1% formaldehyde gel, transferred to nitrocellulose³⁵, and probed with ³²P-labelled nick translated α -chain cDNA⁶. Numbers in the margins indicate M_r (kb).

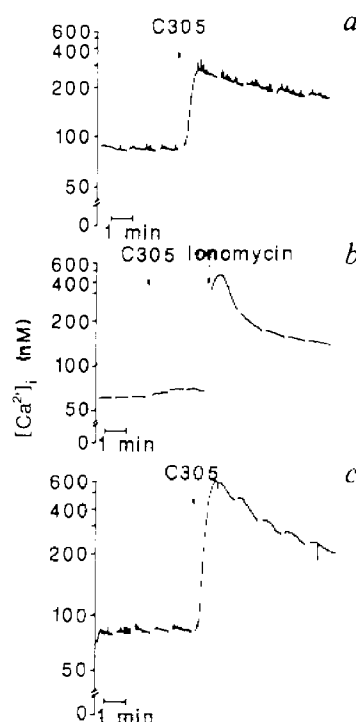


Fig. 6 Changes in $[Ca^{2+}]_i$ in response to anti-receptor (C305) in Jurkat (a); the T3/antigen receptor negative mutant, J.RT3-T3.5 (b); and the β -chain transfectant, PF-2.8 (c). Cells were loaded with the acetoxymethyl ester of quin-2, 20 μ M. Calcium-sensitive quin-2 fluorescence of 10^7 cells per ml was continuously monitored in a Perkins-Elmer 650-40 spectrofluorimeter as previously described³⁷. Interruption in the tracings represent mixture of the cell suspension or addition of C305 (1:300 final dilution of hybridoma culture superantant) or ionomycin, 1 μ M. $[Ca^{2+}]_i$ was calculated according to the method of Tsien *et al.*³⁹.

myristate acetate (PMA). To investigate whether the reconstituted T-cell antigen receptor on the transfected line PF-2.8 was biologically functional, we measured changes in $[Ca^{2+}]_i$ in response to C305 antibody using quin-2, a calcium-sensitive dye. Whereas C305 induced a prompt and sustained rise in $[Ca^{2+}]_i$ in Jurkat (Fig. 6a), it failed to increase $[Ca^{2+}]_i$ in the T3/T-cell antigen-receptor complex negative mutant, J.RT3-T3.5 (Fig. 6b). However, $[Ca^{2+}]_i$ was increased in J.RT3-T3.5 by the calcium ionophore ionomycin (Fig. 6b). When C305 was added to the transfected line PF-2.8, the increase in $[Ca^{2+}]_i$ was restored (Fig. 6c), suggesting that the reconstituted T3/T-cell antigen receptor complex on PF-2.8 was functional. We have demonstrated previously that mutants of Jurkat that fail to express the T3/T-cell antigen receptor complex fail to produce IL-2 in response to PHA plus PMA^{16,37}. Reconstitution of the T3/T-cell antigen receptor complex restored the PHA responsiveness of PF-2.8 as detected by IL-2 production (data not shown). Thus, the transfection of the β -chain gene resulted in not only reconstitution of the expression of the T3/T-cell antigen receptor structural proteins, but also in a functional receptor.

Discussion

The absence of 1.3-kb β -chain transcripts associated with diminished levels of α -chain transcripts could be explained by a defect in a common regulatory element. In this case, however, the presence of a β -chain transcript regulated by the extraneous promoter should not be able to compensate for a defective regulatory protein at the normal α -chain locus. Alternatively, expression of the β -chain may effect the transcriptional level of the α -chain. Results presented here (Fig. 5) provide support for the latter alternative as transfection of the β -chain cDNA into J.RT3-T3.5 results in an increased appearance of α -chain transcripts. Additionally, because the 1.0-kb β -chain transcripts are made in mutants that fail to contain 1.3-kb β -chain transcripts, it is likely that the genes encoding these transcripts have separate regulatory control.

The reconstitution of the T3/T-cell receptor complex using β -chain cDNA suggests that expression of both α - and β -chain proteins is a requirement for the cell-surface expression of T3 proteins. This is in general agreement with the observation that

the cell-surface appearance of T3 on immature T-leukaemic lines and the thymus is coordinated with the appearance of the T-cell receptor heterodimer².

This intimate association between T3 and the T-cell heterodimer may serve as both a structural and functional molecular complex in T-cell activation. We have demonstrated previously that the T3/T-cell antigen receptor complex initiates activation by mechanisms that lead to increases in cytoplasmic free calcium³⁷⁻³⁹. Triggering the T3/T-cell antigen receptor complex results in polyphosphoinositide metabolism and subsequent release of inositol trisphosphate, which in turn induces a rise in cytoplasmic free $[Ca^{2+}]_i$ by the release of intracellular $[Ca^{2+}]_i$ stores⁴⁰. Thus, the association of T3 and the T-cell antigen receptor may represent both a structural interaction necessary for cell-surface expression and a functional interaction necessary for the mechanisms responsible for increases in cytoplasmic free $[Ca^{2+}]_i$. It is clear that the disulphide-linked heterodimer is involved in an antigen binding function. The role of T3 has not been defined, but we suggest that its function is to initiate activation by causing turnover of polyphosphoinositides and mobilization of intracellular calcium.

Finally, the reconstitution of a biologically active receptor by DNA transfer provide direct evidence that β -chain mRNA encodes a protein that constitutes part of the T-cell receptor, and these data provide a basis for further examination of T3/T-cell antigen receptor as a recognition and signal transduction molecular complex.

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Skyhook gravitational-wave detector

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Gravitational waves in the 10–100-mHz band are inaccessible to Earth-based detectors because of seismic noise. Hitherto, the most sensitive detectors in this frequency band have been the Doppler tracking of interplanetary spacecraft¹ and excitations of seismic motions in the Earth's surface^{2,3}. Here we propose a new and more sensitive type of Earth-orbiting gravitational-wave detector, called a 'skyhook', which would operate in the 10–100 mHz band. The skyhook would consist of two masses, one on each end of a long thin cable with a spring at its centre. As it orbits the Earth, the cable would be stretched radially by the Earth's tidal gravitational field. Gravitational waves would pull the masses apart and push them together in an oscillatory fashion; their motion would be transmitted to the spring by the cable; and a sensor would monitor the spring's resulting motion. (An analogous skyhook, for non-gravitational-wave purposes, was proposed by Colombo⁴ *et al.* 10 years ago and is planned for flight on the space shuttle⁵).

The Doppler tracking¹ of interplanetary spacecraft and seismic motions^{2,3} have r.m.s. noise of $h \sim 3 \times 10^{-13}$ in each case. Hellings has pointed out to us that the evaluation of the h sensitivity of seismic motions in his ref. 1 is incorrect by a factor of $\sim 6\pi$ (Earth radius)/(sonic wavelength). We express the noise level of a gravitational-wave detector in terms of $h = f^{1/2}h(f)$, where $h(f)$ is the square root of the spectral density of the transverse-traceless part of the metric perturbation at frequency f . At the end of this century expensive laser interferometric detectors^{6,7} might fly in space with noise levels $h \sim 10^{-21}$. The skyhook could be a relatively inexpensive and unsophisticated broad-band, Earth-orbiting detector, with sensitivity $h \sim 3 \times 10^{-17}$, intermediate between the present detectors and the envisioned interferometric systems.

By integrating for several weeks, the skyhook could detect the gravitational waves from binary stars with periods of order 1 min and wave amplitudes of the order of 10^{-19} , including, for example, the waves that are predicted to come from Geminga according to the Bisnovatyi-Kogan model⁸ (0.6 $M_\odot$ white dwarf in a 1-min period orbit around a 5 $M_\odot$ black hole at a distance 100 pc from Earth) or the Nulsen-Fabian⁹ model (two neutron stars in a several-minute-period orbit at 10-pc distance).

Illustrative parameters for the skyhook are: the masses on the cable ends might be $m_0 = 20$ kg. The cable might have a length $L = 25$ km, diameter $d = 0.6$ mm, and cross-sectional area $S = 0.0028$ cm². It might be made of a composite material with thermal expansion coefficient $\alpha = 10^{-7}$ K⁻¹, density $\rho = 3$ g cm⁻³, and Young's modulus $Y = 1 \times 10^{12}$ dyn cm⁻². In this case, the cable's mass would be $m_c = \rho SL = 21$ kg, its speed of longitudinal sound waves would be $v_s = (Y/\rho)^{1/2} = 5.8 \times 10^5$ cm s⁻¹, and the rigidity of its top half (or bottom half) would be $K_c = 2YS/L = 2.3 \times 10^3$ dyn cm⁻¹. The spring at the cable's centre might have a rigidity for each half (top or bottom) of $K_s = 2.3 \times 10^3$ dyn cm⁻¹. Then the effective mass of each half of the system would be $m = m_0 + m_c/4 = 25$ kg, the net spring constant of each half of the system would be $K = K_c K_s / (K_c + K_s) = 1,200$ dyn cm⁻¹, the angular eigen frequency of the system would be $\omega_0 = (K/m)^{1/2} = 0.22$ s⁻¹, and the eigen frequency would be $f_0 = \omega_0/2\pi = 35$ mHz. Internal dissipation in the cable and spring might give the eigen oscillations a quality factor $Q = 10^5$. The tidal force on each half of the system relative to its centre, augmented by the centrifugal force due to the rotation of the skyhook as it orbits the Earth, would be $F_t = m(L/2)3\Omega^2 = 9.4 \times 10^4$ dyn, where $\Omega = 1.0 \times 10^{-3}$ s⁻¹ is the

skyhook's orbital angular velocity around the Earth. This tidal force would stretch the spring by an amount $x = 2F_t/K_s = 82$ cm.

A gravitational wave with amplitude $h \sim 10^{-17}$ and frequency $f = 2\pi\omega$ in the 10–100-mHz band would produce oscillatory changes of spring length with magnitude $\Delta x \sim m\omega^2 h L / 2K_s \sim 10^{-11}$ cm; this is large enough for a simple sensor to read it out in a time $< 1/\omega \sim 5$ s. Thus, the detector would have a broad-band response.

The length of the cable is limited by the demand that it transmit the gravitational-wave force directly to the spring without excitation of cable sound waves. This requires that the cable length L be equal to about half the sonic wavelength at the highest gravity-wave frequency of interest, $f_{\max}$: that is, $f_{\max} = v_s/2L \approx 100$ mHz. The detector would still have some sensitivity at frequencies higher than this, but its response would be debilitated and complicated by the cable's sound waves.

Six noise sources look as though they might produce serious problems for the skyhook: Nyquist noise produced by the fluctuating part of the skyhook's internal dissipation; fluctuations in cable length produced by fluctuations in solar and Earth heating; fluctuation forces due to the gravity gradients of the high harmonics of the Earth; fluctuating electrical and magnetic forces; micrometeoroid impacts; and atmospheric drag.

The spectral density of the Nyquist force acting on each half of the spring is $S_F = 4mkT\omega_0/Q$, where k is Boltzman's constant and $T = 300$ K is the skyhook's temperature. As the spectral density of the gravity-wave force on each half of the spring is $S_F = [m(L/2)\omega^2 h(f)/2]^2$, with $f = \omega/2\pi$, the Nyquist noise expressed in gravitational-wave units is

$$h_{\text{Nyq}} = f^{1/2}h(f) = \left(\frac{32}{\pi} \frac{kT\omega_0}{mL^2\omega^3 Q} \right)^{1/2} = 3 \times 10^{-17} \left(\frac{f}{30 \text{ mHz}} \right)^{-3/2}$$

The cable is continually being heated by radiation from the Sun and Earth (energy flux $F = 1.7 \times 10^6$ erg cm⁻² s⁻¹) and cooled by emission of radiation into space. If the cable is painted white so that its albedo is $A = 0.97$, and if its specific heat is $C = 6 \times 10^6$ erg g⁻¹ K⁻¹, then its thermal response time (e -folding time for temperature to return to equilibrium after a sudden change of irradiating flux) is $t_T = \pi C \rho T d / 32 F (1 - A) = 600$ s. A fractional oscillation $\Delta F/F$ in irradiating flux at angular frequency ω will produce a fractional oscillation $\Delta T/T = (1/4\omega t_T) \Delta F/F = (1/500)(30 \text{ mHz}/f) \Delta F/F$ in the cable's temperature T . This, in turn, will produce a fractional oscillation $\Delta L/L = \alpha \Delta T = 6 \times 10^{-8} (30 \text{ mHz}/f) \Delta F/F$ in the cable's length, which, in turn, will produce an oscillating tidal force on the top or bottom half of the cable $\Delta F_t = 3m\Omega^2 \Delta L/2$. By equating this with the corresponding gravitational-wave force, we see that the noise produced by fractional fluctuations $\Delta F/F$ in irradiating flux at frequency f is $h_{\text{heat}} = 6(\Omega/\omega)^2 (\alpha T/4\omega t_T) \Delta F/F = 1 \times 10^{-11} (30 \text{ mHz}/f)^3 \Delta F/F$. Thus, if the skyhook is to have a sensitivity of $h \sim 3 \times 10^{-17}$ at $f \sim 30$ mHz, the fractional fluctuations in irradiating flux at 30 mHz and in a bandwidth of order 30 mHz must either be below 3×10^{-6} or be monitored by an instrument onboard the skyhook to this sensitivity.

There are no data with the required accuracy on fluctuations in the solar luminosity in the 10–100-mHz band, but at 1 mHz the ACRIM (Active Cavity Radiometer Irradiance Monitor) detector on the SMM (Solar Maximum Mission) spacecraft reveals¹⁰ $\Delta F/F = 30 \times 10^{-6}$, and as one moves upward in frequency by a factor 30, $\Delta F/F$ may well drop off¹¹ by a factor of 10, to 3×10^{-6} . However, for safety one might wish to monitor the Sun's irradiating flux.

Fluctuations in the Earth's surface temperature and albedo with spatial wavenumber $k = 1/40$ km will produce, as the skyhook orbits the Earth with speed 8 km s⁻¹, fluctuations $\Delta F/F$ at frequency 30 mHz. If the skyhook is at an altitude z above the Earth's surface, $\Delta F/F$ will be reduced below the fractional fluctuations of Earth's temperature and albedo by a factor $\sim \exp(-kz)$, which is $< 10^{-6}$ for altitudes > 550 km. Thus, for orbital altitudes above 550 km, fluctuations in the Earth's radiation are probably not a problem.

The radiative heating of the cable will also vary due to fluctuations in the direction of the cable. However, it seems unlikely that anything will produce fluctuations on the relevant timescale large enough to be a problem.

Of course, whenever the skyhook moves into and out of the Earth's shadow its temperature will change by $\Delta T/T \sim 1/3$; and afterward the cable will require a time of order $t_f \ln 10^8 \sim 3.1$ h to settle back down to a length sufficiently steady for gravity-wave observations. As the orbital period is only ~ 1.7 h, gravity-wave observations will be possible only from a high-inclination orbit where there are long stretches of non-shadow time.

Heating and cooling due to passage through the shadow will probably enhance sudden strain releases (creep) in the cable, and the presence of such creep may necessitate doing experiments with two or more skyhooks simultaneously and cross-correlating their data. Fortunately, each shadow passage will probably cause some annealing of the cable; and, as a result, creep will probably become less serious as the cable ages.

If the skyhook is flown in too low an orbit, noise from the Earth's gravity gradients will be a problem. Fluctuations, $\Delta\sigma$, in the Earth's surface density of mass with wavenumber $k = \omega/v_{\text{orb}} = (1/40 \text{ km})/(f/30 \text{ mHz})$ will produce a tidal force on the top (or bottom) half of the skyhook $\Delta F_t = \pi m G \Delta\sigma (kL) \exp(-kz)$. Equating this to the gravity-wave force $m\omega^2 hL/4$, we obtain for the gravity-gradient noise in gravitational-wave units, $h_{\text{gg}} = (4\pi G \Delta\sigma / \omega v_{\text{orb}}) \exp(-kz)$. For $\Delta\sigma = 3 \times 10^5 \text{ g cm}^{-2}$ and $f = 30 \text{ mHz}$, a skyhook altitude of $z > 1,000 \text{ km}$ will keep $h_{\text{gg}} < 10^{-17}$. The skyhook could function as a good gravity-wave detector at lower altitudes only after the Earth's gravity gradients on scales $k \sim 1/40 \text{ km}$ have been adequately mapped.

Of course, the skyhook itself could be used to map the Earth's gravity gradients, but only one of their six components, the radial-radial component. The skyhook's sensitivity to this component would be $\Delta\Phi_{\text{rr}} = \omega^2 h/2 \sim 5 \times 10^{-19} \text{ s}^{-2}$ in a bandwidth $\Delta f \sim f \sim 30 \text{ mHz}$, which corresponds to $\sim 3 \times 10^{-18} \text{ s}^{-2} \text{ Hz}^{-1/2}$. This is far more accurate than the $10^{-13} \text{ s}^{-2} \text{ Hz}^{-1/2}$ sensitivity of the best gravity gradiometer being developed for flight in space¹², but that gradiometer would measure all six components of the Earth's tidal field, not just the radial-radial component.

The earth's magnetosphere exhibits $f = 10$ – 100 -mHz standing-wave Alfvénic excitations, called Pc2,3,4 and Pil,2 micropulsations¹³, which will interact with the skyhook to produce debilitating noise. On magnetic field lines which cross the geomagnetic equator at ≥ 3 Earth radii ($L \geq 3$ field lines) these micropulsations are present about 50% of the time and have electrical and magnetic field amplitudes $\Delta B \sim (0.1\text{--}100) \text{ nT} = (10^{-6}\text{--}10^{-3}) \text{ G}$; $\Delta E = [(\text{Alfvén velocity})/c] \Delta B \sim (0.1\text{--}100) \text{ mV m}^{-1}$ (refs 13, 14). On field lines with $L \leq 3$, the $f \approx 10$ – 100 -mHz micropulsations may be less frequent and weaker^{14,18} and presumably the predominant quiet-time fields have $\Delta B \leq 0.03 \text{ nT}$, $\Delta E \leq 0.03 \text{ mV m}^{-1}$.

If the skyhook's cable is a good conductor (resistivity $\mathcal{R} \sim 10^{-5} \text{ ohm cm}$; resistance $R_c = \mathcal{R}L/S \approx 9,000 \text{ ohm}$), then the electromotive force $\mathcal{E} = (v/c)BL \approx 6,000 \text{ V}$ caused by its $v \approx 8 \text{ km s}^{-1}$ motion through the Earth's $B \approx 0.3 \text{ G}$ field, will drive current to flow from the magnetosphere into one end region of the cable, along the cable, and out the other end region. Although the magnetospheric impedance for this flow is not well understood (refs 5, 15 and P. M. Banks, personal communication), it will surely be high enough to force the cable to develop a vertical gradient of charge density such that $\Delta q' = (\text{mean charge per unit length on upper half}) - (\text{mean charge per unit length on lower half}) \gg 10^{-3} \text{ ESU cm}^{-1}$. Such a charge difference, interacting with quiet-time electric-field fluctuations $\Delta E_z \sim 0.03 \text{ mV m}^{-1}$, would produce noise at the level $h_{\text{EM}} \gg 10^{-15}$. Thus, the cable must not be a good electrical conductor.

A nonconducting cable, like any satellite, will charge up to $V_0 \sim (\text{a few } kT_e/e) \sim 1 \text{ V}$ in the plasma rest frame because the electron thermal velocity $(3kT_e/m_e)^{1/2}$ exceeds that of the ions and because of photoelectron emission from the cable's surface^{15,16}. The resulting charge per unit length on the cable will

have magnitude $q' \sim 4 \times 10^{-4} \text{ ESU cm}^{-1}$. This charge (essentially equal on the two halves of the cable) will interact with the vertical gradient (d/dz) of the vertical electric-field fluctuations ΔE_z to produce a stretching of the spring. The stretching force on the top (or bottom) half of the spring will be $F_{\text{EM}} \approx (d\Delta E_z/dz)(L^2/8)q'$. Equating this to a gravitational-wave force $\Delta F = m\omega^2 Lh/4$, we find for the electromagnetic noise in gravitational-wave units

$$h_{\text{EM}} \approx 3 \times 10^{-17} \left[\frac{L(d\Delta E_z/dz)}{0.004 \text{ mV m}^{-1}} \right] \left[\frac{30 \text{ mHz}}{f} \right]^2 \left[\frac{V_0}{1 \text{ V}} \right]$$

If the length scales for the vertical gradients of ΔE_z at quiet times are $\geq 10L = 250 \text{ km}$ and if $\Delta E_z \leq 0.03 \text{ mV m}^{-1}$, then h_{EM} will be $\leq 3 \times 10^{-17}$. However, during some micropulsations h_{EM} will be $\gg 3 \times 10^{-17}$. Thus, it will be necessary to carry on board the skyhook one or more electrical field probes to monitor ΔE_z ; and the skyhook orbit should be chosen to spend as much time as possible in the quiet region $L < 3$, that is $z < 6,400 \text{ km} \times (3 \cos^2 \theta_m - 1)$, where z is the orbital altitude and θ_m is the magnetic latitude.

To recapitulate, the orbital altitude must be $z \geq 1,000 \text{ km}$ to avoid gravity-gradient forces, and $z \leq 6,000 \text{ km}$ to maximize the time spent in quiet E -field regions. The inclination of the orbit to the Earth's Equator, i , must be large enough to permit a reasonable fraction of all orbits to be in full sunlight, $i \geq 90^\circ - \sec^{-1}(1 + z/R_\oplus) \approx [60^\circ \text{ if } z = 1,000 \text{ km}; 30^\circ \text{ if } z = 6,000 \text{ km}]$, but small enough (preferably $i \leq 65^\circ$) to minimize the time spent on noisy field lines with $L \geq 3$.

Approximately once per day the skyhook will encounter a swarm of micrometeoroids with individual masses $m \sim 10^{-13} \text{ g}$.¹⁷ The ~ 10 impacts per second during a ~ 10 min encounter will mimic gravity waves with $h \sim 3 \times 10^{-16}$ at $f \sim 30 \text{ mHz}$. Such impacts may be identifiable by the time development of the data; and they can be anticoincided by cross correlating two skyhooks.

Air molecules striking the cable will transfer to it an average fraction $\mu \equiv (\text{accommodation coefficient})$ of their longitudinal momentum. Correspondingly, vertical 'winds' in the residual atmosphere, with wind velocity fluctuations Δv_w and with scale height H for vertical momentum density, will produce a fluctuating longitudinal drag force which mimics gravitational waves with $h \sim [\mu(\Delta v_w/v_{\text{orb}})(L/8H)F_{\text{drag}}][m\omega^2 L/4]^{-1}$. Here F_{drag} is the total neutral-atmosphere-induced drag on the skyhook. This fluctuating drag may require the skyhook to fly at altitudes $z \geq 2,000 \text{ km}$, where $F_{\text{drag}} \leq 0.02 \text{ dyn}$, $H \geq 1,000 \text{ km}$, and

$$h \sim 3 \times 10^{-17} \left(\frac{\mu}{0.1} \right) \left(\frac{1,000 \text{ km}}{H} \right) \left(\frac{F_{\text{drag}}}{0.02 \text{ dyn}} \right) \left(\frac{\Delta v_w}{2s \text{ m s}^{-1}} \right) \times \left(\frac{30 \text{ mHz}}{f} \right)^2$$

and even then correlation drag fluctuations may require two-skyhook correlations for their removal. Charged-particle drag, which is much more difficult to analyze and is intimately connected to electromagnetic noise, might also be a problem.

Noise due to forces on the cable (drag, micrometeoroid, and electromagnetic) might be strongly reduced by using a more complicated skyhook design (suggestion by R.W.P. Drever): use a single cable (no central spring) with one spring at each end between cable and end mass; make the cable much stiffer than the two springs, $k_c \gg k_s$, so it acts as a rigid rod connecting them; and add the displacements of the springs.

The skyhook is, in summary, an attractive, simple instrument with gravity-wave sensitivity in an interesting range where sources might exist but are not guaranteed to exist.

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The geomagnetic mass spectrometer—mass and energy dispersions of ionospheric ion flows into the magnetosphere

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NASA's Dynamics Explorer (DE) mission was designed to study the coupling between the Earth's magnetosphere, ionosphere and neutral thermosphere¹. One area of major interest is the outflow of ionospheric plasma into the magnetosphere, the scale and significance of which is only now becoming apparent with the advent of mass-resolving, low-energy ion detectors. Here we compare observations of ion flows in the polar magnetosphere, made by the retarding ion mass spectrometer (RIMS)² on DE1, with those made simultaneously in the topside ionosphere by the ion drift meter (IDM)³ on the lower-altitude DE2 spacecraft. The results show the dayside auroral ionosphere to be a significant and highly persistent source of plasma for the magnetosphere. The upwelling ionospheric ions are spatially dispersed, according to both their energy and mass, by the combined actions of the geomagnetic field and the dawn-to-dusk convection electric field, in an effect analogous to the operation of an ion mass spectrometer.

The two DE satellites were launched into co-planar polar orbits on 3 August 1981; DE1 being in a highly elliptical orbit which carried it out to a geocentric distance, r , of 4.5 Earth radii (R_E), while DE2 remained at altitudes below about 1,000 km. During the first 18 months of the mission, the DE1 apogee precessed from the north to the south pole.

The RIMS instrument on DE1 comprises three detector heads, each an ion mass spectrometer with a potential analyser input, and each capable of observing ions of two mass-per-charge ratios simultaneously. Ion species of mass up to 32 AMU can be resolved and for each species an integral energy spectrum over the range 0–50 eV (relative to spacecraft potential) is obtained. The RIMS radial head sweeps through a full range of pitch angles twice during each satellite spin period of 6 s: Fig. 1 shows data from this radial head for a pass from north to the south polar regions on 3 April 1982. By this date, DE1 perigee was close to the geomagnetic equator (time C) and no data were obtained between times B and D, when the ambient

plasma density was too great for RIMS to operate. Figure 1 shows contours of O^+ and H^+ ion counts per accumulation period (12 ms) as a function of time and satellite spin angle. Count rates are approximately proportional to integral ion flux, one count per sample being roughly equivalent to 3.1×10^4 and $4.4 \times 10^4 \text{ cm}^{-2} \text{ s}^{-1} \text{ sr}^{-1}$ for H^+ and O^+ respectively; during most of this pass they were largest when the spin angle was close to zero, that is, when the radial head was looking along the orbit path or RAM direction. This indicates that the plasma was cold and isotropic and that ion flow velocities were much smaller than the spacecraft speed ($\sim 7 \text{ km s}^{-1}$ at $r = 2R_E$ and 10 km s^{-1} at $r = 1.1R_E$)⁴. Exceptions to this were the behaviour of H^+ ions in the northern polar cap (before 1157 UT, the time A), for which the peak count rate was shifted from the RAM direction towards the upward field-aligned direction (the upper of the two dashed lines), revealing upward field-aligned flow, as predicted for the light-ion polar wind^{5,6}; in addition the peak O^+ ion count rates near both magnetic poles (before about 1150 UT and after about 1240 UT) are shifted towards the downward field-aligned direction, indicating the ions are moving downward under the influence of gravity⁷. However, the largest flow features were seen at times A and E when DE1 passed through the morning sector auroral ovals in each hemisphere and strong upward flows were observed of both O^+ and H^+ ions.

These upflows have been termed 'upwelling ion events'⁴ as all ion species show the effects of equal ion heating below the satellite (to parallel and perpendicular temperatures of ~ 2 and 10 eV, respectively) and carry an upward heat flux. Ion species observed streaming upwards in these events include H^+ , He^+ , O^+ , N^+ and O^{2+} (ref. 7), and there is evidence that molecular ions N_2^+ , O_2^+ and NO^+ , seen by RIMS in the polar magnetosphere, may also originate from the events. The number fluxes of upwelling O^+ are very large, requiring of the order of 10^9 ions $\text{cm}^{-2} \text{ s}^{-1}$ out of the topside ionosphere, which is an order of magnitude larger than the cold polar wind flux summed over all ion species^{5,6}: the total O^+ outflow from both hemispheres has been estimated to average over 10^{25} s^{-1} which compares with an estimated polar wind outflow of $5 \times 10^{25} \text{ s}^{-1}$. A statistical survey of 2 years' RIMS data has shown that these events are a highly persistent phenomenon and are only found in a narrow region near the morning and noon polar cap boundaries⁴.

The interaction of the solar wind, and the interplanetary magnetic field (IMF) embedded within it, with the Earth's magnetosphere generates large-scale electric fields which give 'convection' of plasma at high latitudes. If the IMF has a southward component, this flow is anti-sunward over the polar cap.

Solar wind ions injected into the magnetosphere in the dayside auroral zone have been observed to be dispersed in mass, energy and pitch angle by this poleward motion⁸: lower velocity ions are found further poleward because their time of flight is greater and hence they are convected over greater distances. This effect should also be observed for the upwelling ionospheric ions described above, particularly at great altitudes where the dispersion should be larger.

Early in the DE mission, RIMS afforded an excellent opportunity to see dispersion of upflowing ionospheric ions, with DE1 apogee being over the northern polar cap. Figure 2 shows data from 22 October 1981 when DE1 and DE2 were in close magnetic conjunction over the northern dayside auroral region. The level of geomagnetic activity was moderately high ($K_p = 5.0$), for which the occurrence probability of upwelling ions is very close to unity⁴: the IMF was (and had previously been) predominantly southward, giving anti-sunward convection, as was observed by the IDM on DE2.

Figure 2a shows the retarding potentials required to reduce the count rates from the RIMS radial head to below two per sample, for upward field-aligned flows of H^+ , He^+ and O^+ ions near 22,000 km in altitude; these are estimates of the peak energy for each ion species. Figure 2c shows the field-aligned flow velocity and flux, as seen by the IDM during the DE2 pass at an altitude near 900 km. The arrows relate the observations to

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Fig. 1 Spin angle/time plots of count rate contours for O^+ and H^+ ions, observed during a pole-to-pole pass of DE1 on 3 April 1982 by the RIMS radial head. Zero spin angle corresponds to the satellite orbit direction and the upward field-aligned direction is shown by the dashed line in the Southern Hemisphere ($MLAT < 0$) and by the dashed-and-dotted line in the Northern Hemisphere. For each universal time (UT), the geocentric distance (r), magnetic local time (MLT) geomagnetic latitude (MLAT) and invariant latitude (Λ) of DE1 are shown.

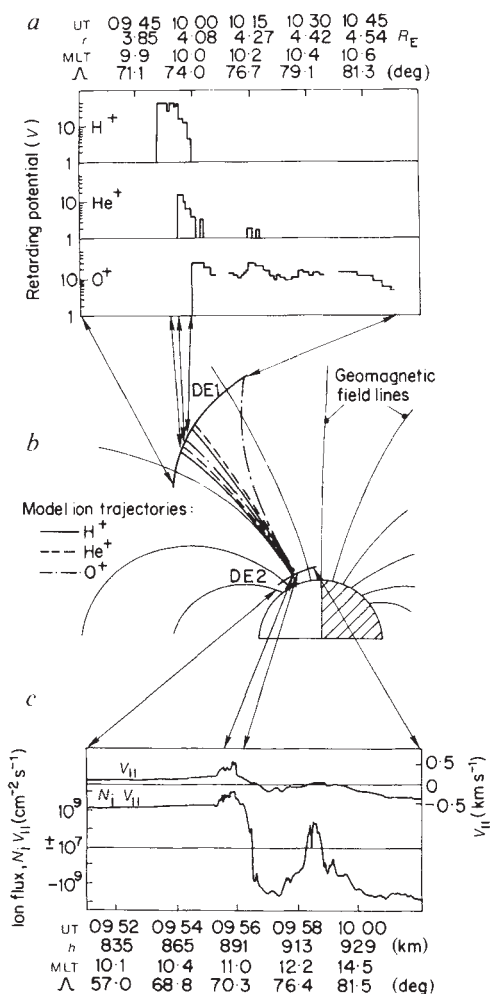
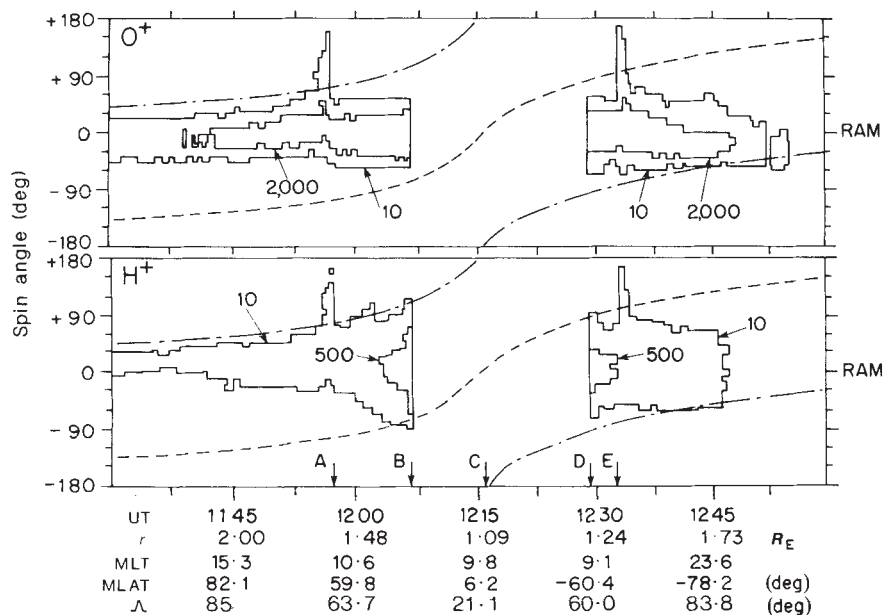


Fig. 2 *a*, Retarding potential required to reduce ion count rates observed by RIMS radial head below two per accumulation period (12 ms) for a high-altitude, polar pass of DE1 on 22 October 1981. *b*, Results for field-aligned flows of H^+ , He^+ and O^+ ions. *c*, Field-aligned ion velocity ($V_{||}$) and the flux ($N_i V_{||}$) observed simultaneously by the IDM on DE2. Positive values are upward. Orbital tracks and model ion trajectories for this period are shown projected onto the moon-midnight meridian in *b*: the ion trajectories shown are computed for the highest and lowest energy ions of each species as observed by RIMS. Λ is the invariant latitude of the spacecraft and h is the altitude.

the locations of the satellites, projected onto the noon-midnight meridional plane in Fig. 2*b*. As DE1 moved polewards, RIMS observed first H^+ , then He^+ and finally O^+ ions at any one energy and, in addition, the peak ion energy decreased for each ion species. Hence these observations qualitatively show the predicted mass and energy dispersions. That these data are quantitatively consistent with the 'geomagnetic mass spectrometer' concept is demonstrated by the model ion trajectories also shown in Fig. 2*b*. These have been computed from the ion energies observed by RIMS using a two-dimensional kinetic model⁹ with a dawn-to-dusk electric field consistent with the IDM observations of field-perpendicular ion flow. It is assumed that the ion motion is collision free and that the electrostatic potential distribution along the geomagnetic field lines of the polar cap is as predicted for the cold polar wind⁵, as has recently been inferred from other RIMS data⁷. It can be seen that all ion trajectories converge to a narrow ionospheric source region where an exceptionally large ion upflow signature was observed by the IDM (total ion flux exceeding $10^9 \text{ cm}^{-2} \text{ s}^{-1}$). Figure 2 shows that O^+ ions were found throughout most of the dayside polar cap where RIMS data were available for this pass of DE1; other passes at high Kp (≥ 5) also reveal O^+ ions throughout the polar caps⁷.

These observations show that the narrow ionospheric source region acts as the collimation slit of the geomagnetic mass spectrometer which can disperse ions, particularly cold and heavy ions, throughout the polar cap; such O^+ ions have already been observed by both ion detectors on DE1^{10,11}. Lastly, note that these ions are present in addition to (and are warmer than) the light ions of the classical cold polar wind, which can only be observed by RIMS at these altitudes with the application of an input aperture bias potential, which overcomes the repulsive effect of the positive spacecraft potential¹².

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Raman intensity measurements for determining conformer populations as a function of pressure

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Raman spectroscopy of fluids under pressure has been increasingly studied^{1,2} and applied^{3,4} over the past few years. Essential to the extension of these investigations is the reliable interpretation of the pressure-induced spectral changes of model systems in terms of variations in conformation and molecular dynamics. Conformational investigations have been made on systems that were suspected of undergoing conformational change at high pressures, for example, *n*-alkanes⁵⁻⁹, haloalkanes¹⁰⁻¹³ and substituted cyclohexanes¹⁴⁻¹⁹. Raman investigations reported here reveal significant pressure-induced spectral changes in both conformationally sensitive and insensitive systems. We suggest that these arise from a breakdown in the tenet of the pressure invariance of a direct proportionality between Raman scattering intensity and concentration. This is most apparent from the differential behaviour of symmetrical and antisymmetrical vibrations. We propose a re-evaluation of some previous interpretations of the pressure-induced spectral changes.

The vibrations of polyatomic molecules are usually discussed in terms of concerted combined motions of atoms, termed normal modes, where each normal mode forms the basis for an irreducible representation of the point symmetry group of that molecule. In general, a molecule with *n* atoms will possess $3n - 6$ normal modes or $3n - 5$ for linear molecules. Corresponding to each normal mode, a so-called normal coordinate *Q* is introduced that allows the various physical features of the molecule (such as potential energy, polarizability and dipole moment) to be represented as a function of a single length dimension.

For conformationally variant molecules, the essential assumption required for effective interpretation of the spectral changes induced by pressure is that the concentration *C* of a given conformer *r* should be directly proportional to a normalized intensity I_{rk}^{obs} for the *k*th normal mode of that conformer.

$$C_r = a_{rk} \cdot I_{rk}^{\text{obs}}$$

where a_{rk} is the proportionality constant.

For Raman spectroscopy, the intensity of an observed vibrational band is, to a first approximation, directly proportional to the derivative of the molecular polarizability (itself a second rank tensor) with respect to the normal coordinate, at the equi-

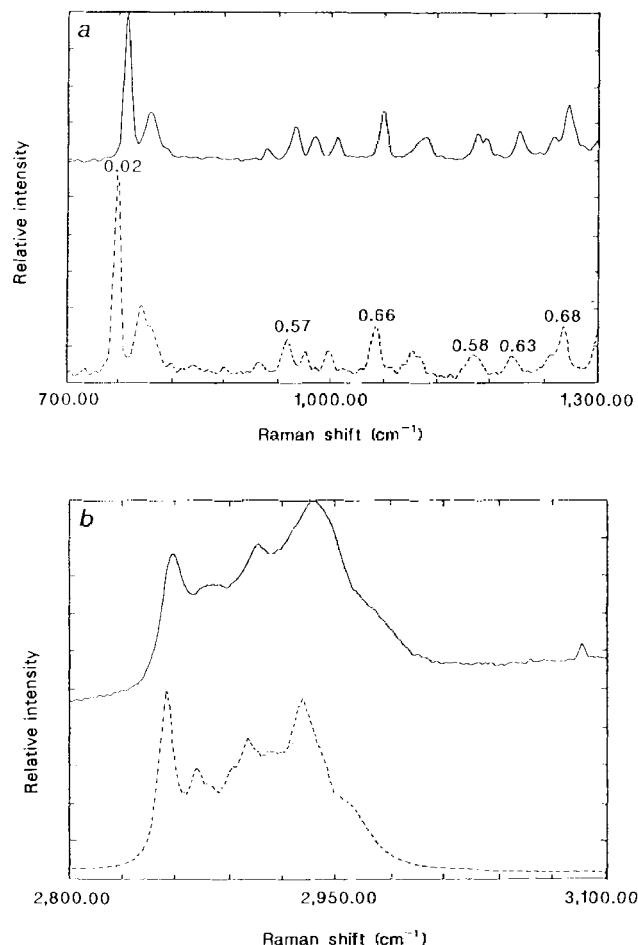


Fig. 1 Raman spectra of *cis*-1,4-dimethylcyclohexane: *a*, optical skeletal region; *b*, C-H, region 180° scattering, 514.5-nm excitation: ---, 0.001 kbar; —, 5.5 kbar. The numbers superimposed on the *a* spectrum are the depolarization ratios corresponding to the major peaks.

lium position. Thus, for the above assumption to hold, the polarizability change corresponding to a given vibrational mode must itself be independent of pressure.

$$(\delta\alpha/\delta Q_k)_0 = \text{constant}$$

where α is the polarizability tensor, Q_k is the normal coordinate for the *k*th vibrational mode, and the subscript 0 implies the differential polarizability change at the equilibrium position.

In most investigations reported, there has been no attempt to verify the above assumption. Indeed, in the only investigation that does attempt this⁹, it is not clear that the chosen reference bands possess a sufficiently dissimilar character and symmetry to be broadly representative.

We have carried out some detailed high-pressure Raman investigations of a wide range of fluids (see Table 1). We included a comprehensive set of disubstituted cyclohexanes to allow a systematic assignment of some of the spectral features to particular conformational species. As a test of any features assigned in conformationally variant systems, for example, fluids F3, F4 and F7, comparisons were to be made with related conformationally invariant systems, for example, fluids F2, F5 and F6.

We have performed extensive tests to ensure that we have eliminated all contributions deriving from stress-induced birefringence and other optical artefacts. We used two separate Raman microscope systems, scanning and multichannel, and two different diamond anvil cells in addition to a traditional hydrostatic equilibrium cell¹⁹. In all cases, the essential observations were the same.

Table 1 Fluid number and composition

Fluid no.	Composition
F1	Methyl cyclohexane
F2	<i>cis</i> -1,2-dimethylcyclohexane
F3	<i>trans</i> -1,2-dimethylcyclohexane
F4	<i>cis</i> -1,3-dimethylcyclohexane
F5	<i>trans</i> -1,3-dimethylcyclohexane
F6	<i>cis</i> -1,4-dimethylcyclohexane
F7	<i>trans</i> -1,4-dimethylcyclohexane
F8	Toluene
F9	Toluene d-8
F10	Cyclohexylmethanol
F11	Methyl cyclohexane carboxylate
F12	Methyl cyclopentane carboxylate
F13	Ethyl hexanoate
F14	2,4-dicyclohexyl, 2-methylpentane

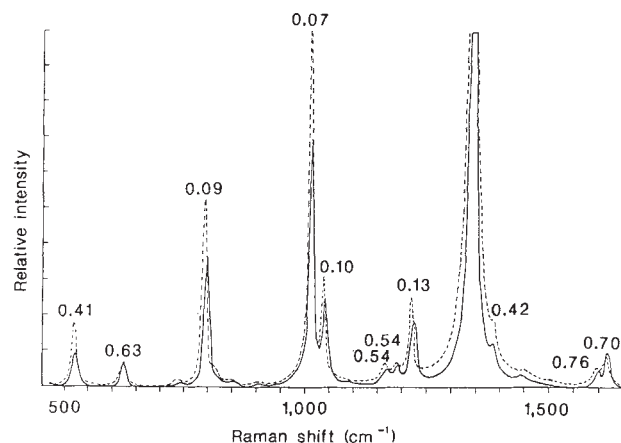


Fig. 2 Raman spectra of toluene, skeletal region, 180° scattering, 514.5-nm excitation: ----, 0.001 kbar; —, 12.8 kbar.

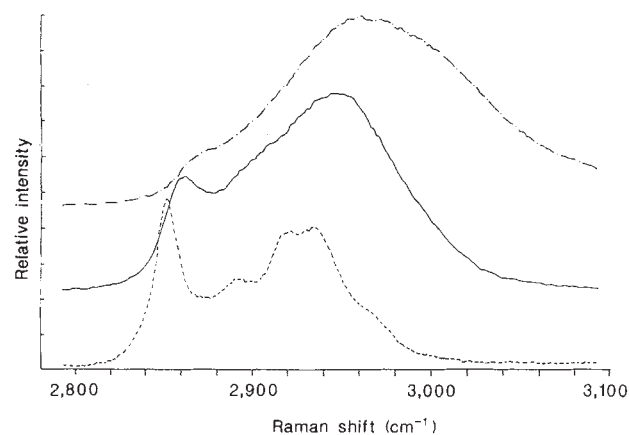


Fig. 3 Raman spectra of 2,4-dicyclohexyl-2-methylpentane, C-H region 180° scattering, 514.5-nm excitation: ----, 0.001 kbar; —, 17.3 kbar; - · - · -, 40.2 kbar.

Examples typifying our observations come from two conformationally invariant systems, notably *cis*-1,4-dimethylcyclohexane (F6) and toluene (F8). Figures 1a and 1b depict the ambient and high-pressure (5.5 kbar) Raman spectra of F6 in the optical skeletal (OS) and C-H stretching regions. The numbers superimposed on the OS spectra are the depolarization ratios corresponding to the major features. Figure 1a shows the decrease in integrated intensity of the 765 cm⁻¹ ring breathing band ($\rho = 0.02$) relative to the remaining bands with depolarization ratios in the range 0.57–0.68. In the C-H stretching region (Fig. 1b), the effect is more marked with the 2,855 cm⁻¹ symmetrical CH₂-stretching mode dramatically losing intensity relative to the antisymmetrical 2,931 cm⁻¹ band.

Figure 2 shows toluene, ambient and high-pressure spectra with the corresponding assigned depolarization ratios. Several features are apparent, most notably the loss in relative integrated intensity of the 1,004 cm⁻¹ (A_1 , $\rho = 0.07$), 1,030 cm⁻¹ (A_1 , $\rho = 0.10$) and 1,208 cm⁻¹ (A_1 , $\rho = 0.13$) bands compared with the 1,155 cm⁻¹ (B_1 , $\rho = 0.54$), 1,178 cm⁻¹ (A_1 , $\rho = 0.54$) doublet, and also the 521 cm⁻¹ (A_1 , $\rho = 0.41$) compared with the 623 cm⁻¹ (B_1 , $\rho = 0.73$) bands.

Finally, in a more complex molecule, 2,4-dicyclohexyl-2-methylpentane, originally suspected of conformational change at high pressures³, Fig. 3 illustrates the loss of intensity of the CH₂-symmetrical mode (2,850 cm⁻¹) compared with the CH₂-antisymmetrical region (2,930 cm⁻¹) with its virtual disappearance at pressures approaching 50 kbar.

These examples reveal the relationship between pressure-induced intensity reduction and the symmetry, and hence depolarization ratio, of a given Raman band. Therefore, in many instances one observes that ($I_{\text{symm}}/I_{\text{asymm}}$) decreases as pressure increases. However, this effect is most marked for the C-H stretching region. Further observations may be noted for Raman bands of similar depolarization ratios but different vibrational assignments. For example, the C=C bands of a range of unsaturated ester fluids (not listed) were significantly suppressed relative to the C=O stretching bands, despite possessing similar depolarization ratios (~ 0.25).

A qualitative explanation for these observations is available in that the loss of intensity of a given vibrational band as a function of pressure is directly related to the volume change associated with that vibration. Thus, symmetrical modes, in general, and C-H stretching modes, in particular, with large associated volume changes are expected to be suppressed at elevated pressures.

Considerable attention has been given to the effect of pressure on vibrational frequencies of molecular systems. In general, the positive frequency shifts observed with increasing pressure are explained in terms of decreases in equilibrium bond lengths and consequent increases in bond force constants, related to the anharmonicity of the vibrational potential well^{20,21}. In contrast,

very little theoretical attention has been focused on the effect of pressure on vibrational band intensities, particularly in fluids, despite the fact that it is well known that in studies of vibrations in molecular crystals symmetrical modes are suppressed relative to the liquid state and are further suppressed by pressure^{22–24}. It is apparent that as pressure induces significant changes in molecular bond lengths such as to cause appreciable force constant changes and consequent vibrational frequency shifts, the shape and slope of the polarizability/normal coordinate curves themselves must become altered. The change in ($\delta\alpha/\delta Q_k$)₀ thus induced must cause intensity changes. This will, therefore, negate the original assumption of a pressure invariance of the concentration/intensity relationship.

These observations raise additional explanations for reported pressure-induced spectral changes. As an example of one of the most thorough conformational investigations reported, one may consider the study by Wong *et al.*⁹ of the longitudinal acoustic mode (LAM) region of the Raman spectrum of *n*-hexane. Their most striking observation was of a considerable reduction in intensity of the band caused by the all-*trans* (*ttt*) conformer relative to bands resulting from the other conformers containing one or two gauche arrangements. However, the key (*ttt*) band used in the population measurement corresponds to a highly symmetrical concertina-like vibration of the molecule with a large associated molecular volume change. In contrast, the remaining bands of this LAM region, whose relative normalized intensities did not vary so significantly, correspond to vibrations of significantly lower symmetry and smaller associated volume change. Our observations on conformationally invariant systems suggest that their tenet of direct proportionality between intrinsic intensity and concentration is not upheld, particularly considering vibrations of such dissimilar symmetry and associated volume change.

Some recent infrared investigations²⁵, again including studies of some conformationally invariant systems, have revealed a similar character of spectral changes as a function of pressure to those observed by Raman spectroscopy. Assignment of the infrared bands to symmetrical and antisymmetrical vibrations is much more difficult, but notably in the C-H region where the intensity changes are most marked, the general behaviour is identical to the Raman data presented here. Thus, our main assertions have wider application to all vibrational spectroscopic studies of fluids under pressure.

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¹⁰Be in ice at Vostok Antarctica during the last climatic cycle

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We have previously reported accelerator mass spectrometer measurements of the cosmogenic isotope ¹⁰Be (half life 1.5 Myr) in an 906-m ice core recovered at Dome C, Antarctica¹. In addition to an enhanced concentration during a period of reduced solar activity (Maunder minimum, AD 1645-1715)², we found substantially increased concentrations during that part of the core corresponding to the last ice age. Both effects have been confirmed and extended in Greenland ice cores^{3,4}. The recovery of a 2,083-m ice core at Vostok, Antarctica⁵, together with an extended oxygen isotope chronology reported elsewhere in this issue⁶, permits an extension of ¹⁰Be studies over the whole of the last climatic cycle (~125 kyr). We report here such measurements, which show an excellent correlation with the oxygen isotope and thus, presumably, climate record over the whole of this period. The results imply that precipitation rates in the Antarctic during the last interglacial were similar to those of the Holocene, but were roughly halved during the last glaciation.

A total of 22 samples, weighing from 0.7 to 2.0 kg, were available for the ¹⁰Be study. After the addition of 0.5 mg of ⁹Be carrier, the samples were treated as described previously¹. The ¹⁰Be measurements were made on a General Ionex Tandemtron accelerator mass spectrometer system, using the procedure described elsewhere⁷. The uncertainties have been calculated using a 7% instrumental uncertainty (conservatively estimated on the basis of the reproducibility of a standard and several of the ice samples), together with 1 s.d. statistics on the number of ¹⁰Be events counted. Because we consider that the chronology of the core is still uncertain, no corrections for decay of ¹⁰Be have been made. However, using the present best estimate of the age of the ice (see below), such a correction would be <7% for the deepest sample measured. The results are shown in Fig. 1, together with the oxygen isotope profile for the same core⁶ (note that the ¹⁰Be concentration scale has been inverted to facilitate comparison with the ¹⁸O record). The correlation between the two curves is remarkable. Not only does the ¹⁰Be concentration return to Holocene levels from 1,650 to 1,875 m (believed to represent the last interglacial), but the coldest periods during the glacial, as indicated by the oxygen isotope profile, are also reflected by maxima in ¹⁰Be concentrations.

We have previously discussed three possible explanations for ¹⁰Be concentration variations in ice¹. One involves production

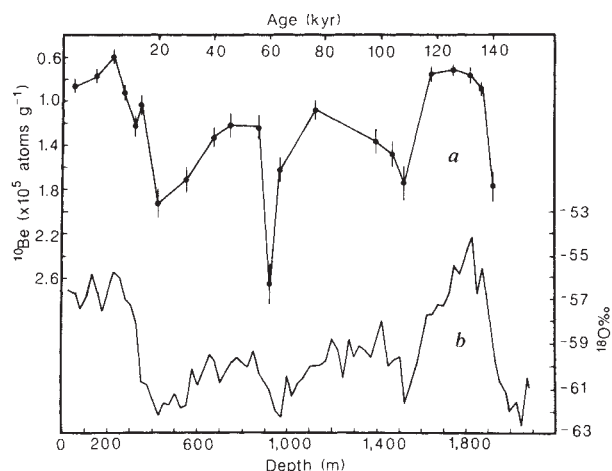


Fig. 1 Concentrations of ¹⁰Be (a) and ¹⁸O (b) (from ref. 6) as a function of depth in ice at Vostok. Note inverted scale for ¹⁰Be concentration to facilitate comparison of the two curves. Time scale is that adopted in ref. 6.

variations, possibly induced by solar modulation effects; the second would result from changes in atmospheric circulation; and the third links the concentration variations to changes in precipitation rates. Although we cannot yet exclude effects caused by the first two factors, the present results reinforce our belief that the third mechanism is the most probable explanation for the observed long-term correlation of ¹⁰Be with climate. Indeed, if one can neglect, or correct for, ¹⁰Be flux variations, ¹⁰Be concentrations may be used to estimate past precipitation rates^{8,9}. Such a calculation is shown in Fig. 2, using the data of Fig. 1 and assuming that the deposition flux of ¹⁰Be at Vostok has, on average, been constant over the last climatic cycle.

This interpretation of the ¹⁰Be results implies that the precipitation rate at Vostok during the last ice age was ~50% of that during the present and previous interglacial. This is consistent with results based on conductivity measurements of annual layers in the Byrd core, which indicate about a factor of 2.5 decrease going from the early Holocene to the late glacial period (C. Hammer, personal communication). It is also in partial agreement with the results of Wilson and Hendy who deduced a decrease in annual layer thickness during the late glacial period at Vostok based on sodium variations¹⁰. However those authors also found a precipitation rate larger than the Holocene value at ~900 m which is not indicated by the ¹⁰Be results. Thompson *et al.*¹¹, using annual layers derived from particle concentrations, found an approximately constant precipitation rate during the late glacial period and Holocene at Dome C. As mentioned earlier, our own ¹⁰Be results at Dome C¹ suggest about the same reduction in precipitation during the glacial period as at Vostok.

We have previously pointed out¹ that a factor of two decrease in precipitation during the late glacial period at Dome C is consistent with the hypothesis that precipitation on the Antarctic plateau has been limited by the saturated water vapour pressure above the inversion layer¹², as estimated from ¹⁸O values in the ice. That this observation also holds at Vostok can be seen in Fig. 2, where a precipitation rate based on vapour pressure⁶ is compared with that estimated from the ¹⁰Be. In fact, the agreement of the two curves indicates that the inversion temperature may have been the dominant factor in determining precipitation rates on the Antarctic plateau during the past climatic cycle. This hypothesis is the basis for the adoption by Lorius *et al.*⁶ of a model where the past accumulation rate is assumed to be correlated with temperature, as deduced from oxygen isotope analysis. Several possible explanations why ¹⁰Be may be anti-correlated with precipitation rates in polar regions are discussed in ref. 9.

The chronology that follows from a reduced accumulation rate during the glacial period, together with a flow model described in ref. 6, is shown in Fig. 1. Although this timescale

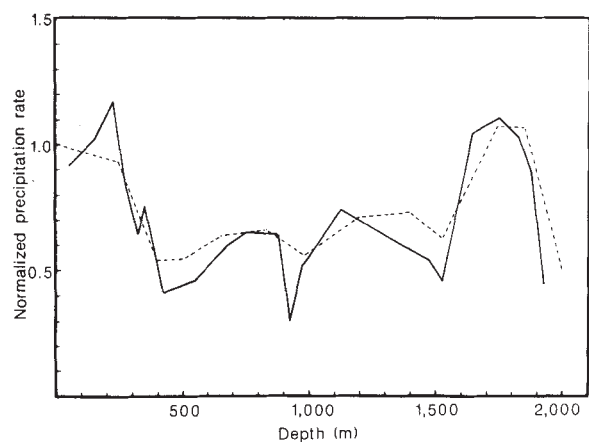


Fig. 2 Estimated precipitation rate (normalized to Holocene value) at Vostok in the past assuming a constant ^{10}Be deposition flux (solid line) and precipitation controlled by saturated water vapour pressure at temperature of inversion layer (dashed line, from ref. 6).

appears reasonable, and correlates with several other parameters⁶, it is obvious that a confirmation by direct measurement of the age of the ice at some point in the ice age is desirable. We hope to make such a measurement in the future using an accelerator mass spectrometry determination of the ^{14}C contained in the ice.

The only significant deviation between the ^{10}Be and ^{18}O curves of Fig. 1 is in the ^{10}Be measurement at 925 m. The ^{10}Be concentration in this sample is nearly 50% larger than any other, and does not correspond to the ^{18}O minimum. It is dangerous to construct any hypothesis on the basis of a single point, but it is conceivable that this sample may be showing enhanced ^{10}Be for reasons similar to that during the Maunder minimum, that is, decreased solar modulation. We plan to make more detailed measurements around this point.

In conclusion, although we do not exclude the possibility of production changes, the 'first order' cause of ^{10}Be concentration variations in Antarctic ice during the last climatic cycle seems to result from precipitation rate variations. ^{10}Be measurements may thus be a useful way of determining these precipitation rates and helping to establish chronologies. The present work suggests that the precipitation rate in the Antarctic during the last interglacial was similar to the Holocene, whereas during the ice age it was about 2× lower. The good correlation between ^{10}Be and temperatures deduced from oxygen isotopes also suggest that temperature has been the dominant factor controlling precipitation on the Antarctic plateau for at least the past ~150 kyr.

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Net flux of carbon dioxide from tropical forests in 1980

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Deforestation in the tropics is responsible for an annual net release of carbon to the atmosphere, estimated for 1980 at between 0.5 and 4.2×10^{15} g (refs 1–6). By comparison, the release of carbon from combustion of fossil fuels was 5.2×10^{15} g in 1980. The wide range of estimates for the tropical biota and soils has been due primarily to different estimates of the rate of deforestation^{2,7}. A recent assessment of the world's tropical forests by the Food and Agriculture Organization/UN Environment Program^{8,9} provides a comprehensive, country-by-country survey of deforestation in the late 1970s as well as estimates of the volumes of wood in tropical forests. The assessment thus provides an independent data base, perhaps the most reliable to date, from which terrestrial releases of carbon to the atmosphere can be calculated. Here we compare the FAO/UNEP survey with other sources of data on rates of deforestation and stocks of carbon in the tropics; then we present the calculated net flux of carbon between tropical forests and the atmosphere based on these different data; and, finally, we consider the accuracy of current estimates of the net biotic flux.

The FAO/UNEP's^{8,9} estimate of deforestation in the tropics is compared in Table 1 with estimates made by Myers^{10–12} and the FAO's Production Yearbook¹³. Comparison of the rates given by FAO/UNEP and the Production Yearbook assumes that deforestation of the open and closed forests of the former is equivalent to the net decrease of the forests and woodlands of the latter. Because the Production Yearbooks include fallow forests in their category of forest and woodland, while

Table 1 Annual rates of deforestation of tropical forests (1975–80) according to three sources (10^6 hectare yr^{-1})

	Closed and open forests		Closed forests	
	FAO/UNEP ⁸	Production Yearbook ¹³	FAO/UNEP ⁸	Myers ^{10,11*}
America	5.391	5.430	4.119	2.67
Africa	3.678	3.089	1.333	1.57
Asia	2.005	0.895	1.815	3.27
All tropics	11.074	9.414	7.267	7.51

* About 36% of the total conversion of humid forests to agriculture and pasture was from non-fallow forests^{10,11,14}. The rest was from fallow forests, not included in this table. About 20% of the conversion of forests due to fuelwood extraction was of non-fallow forests. These 0.5×10^6 hectare yr^{-1} were divided equally among regions.

FAO/UNEP does not include the deforestation of fallow, the comparison is not strictly valid. Nevertheless, it shows agreement for the entire tropics within 8% of the mean (Table 1).

Comparison of Myers' estimate with that of FAO/UNEP is only possible for closed forests, because Myers did not include dry forests or woodlands in his survey. Furthermore, Myers included the clearing of fallow as well as primary forests. The primary forests of Myers correspond to the primary, secondary, and logged forests of FAO/UNEP. His secondary forests correspond to fallow forests in the terminology of FAO/UNEP. When Myers' estimates of total conversion are partitioned between primary and secondary forests on the basis of the distribution

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Table 2 The rates (10^6 hectare yr^{-1}) of conversion of fallow and non-fallow forests to permanently and temporarily cleared land; the rates are for 1980 and apply to closed forests of the humid tropics

	Non-fallow forests			Fallow forests			Total conversion
	Permanent	Temporary	Total	Permanent	Temporary	Total	
Pasture	0.7	0	0.7	1.3	0	1.3	2.0
Fuelwood	0.5	0	0.5	2.0	0	2.0	2.5
Agriculture	3.9	1.9	5.8	6.8	3.4	10.2	16.0
Total	5.1	1.9	7.0	10.1	3.4	13.5	20.5

Two and three significant figures are for clarity and consistency and do not indicate the level of accuracy. Myers¹¹ estimated that 90 and 50 million of the subsistence agriculturalists occupied fallow and non-fallow forests, respectively (Myers' terminology of primary and secondary forests is equivalent to the terminology of non-fallow and fallow forests used here). The annual rates of deforestation due to subsistence agriculture (16.0×10^6 hectare yr^{-1})¹² and to ranching (2.0×10^6 hectare yr^{-1}) were assumed to be distributed between non-fallow and fallow forests in proportion to these numbers of people. The proportions of non-fallow and fallow forests cleared as a result of fuelwood harvest (2.5×10^6 hectare yr^{-1}) were 20% and 80%, respectively¹⁴.

Table 3 Carbon in vegetation and soils of different land-use categories in the world's major tropical regions

Tropical region	Type of forest	Carbon in vegetation* (10^3 kg hectare $^{-1}$)			Carbon in soils (10^3 kg hectare $^{-1}$)		
		Undisturbed forest	Mature fallow	Agriculture	Undisturbed forest	Mature fallow	Agriculture
America	Moist	82 (176)	33 (70)	5	100	90	70
	Seasonal	85 (158)	34 (63)	5	100	90	70
	Dry	27 (27)	11 (11)	5	69	62	48
Africa	Moist	124 (210)	50 (84)	5	100	90	70
	Seasonal	62 (160)	25 (64)	5	100	90	70
	Dry	15 (90)	6 (36)	5	69	62	48
Asia	Moist	135 (250)	90 (90)	5	120	108	84
	Seasonal	90 (150)	50 (50)	5	80	72	56
	Dry	40 (60)	35 (35)	5	50	45	35

* Values outside parentheses are derived from volumes of growing stock^{8,9}. Values inside parentheses are based on direct measurements of carbon stocks¹⁷.

of subsistence agriculturalists^{11,14}, his rates of deforestation of primary forests agree well with the FAO/UNEP estimate for closed forests (Table 1). Agreement is within 3% of the mean for all the tropics, but within 60% of the mean for Asia. If Myers' estimates of deforestation apply strictly to tropical, moist, broad-leaved forests, his estimate is ~25% higher for the entire tropics than the FAO/UNEP estimate based on the same criteria¹⁴.

The major difference between the surveys of Myers and FAO/UNEP is not their estimates of current rates of deforestation, however, but their assumptions concerning the fate of fallow lands. The traditional form of agriculture in the tropics, shifting cultivation, has been largely replaced in recent years by a more permanent type of agriculture, referred to as "forest farming" by Myers¹⁰⁻¹² or as "sedentary shifting cultivation" by the US National Research Council (NRC)¹⁵. Even where the cultivators themselves are shifting, the land may remain permanently cleared by the arrival of new colonists or by the inability of forests to re-establish after intensive use of the land. The FAO/UNEP study, although recognizing the process, did not address the deforestation of forests that had been previously in agriculture (fallow forests) because such forests were not considered a timber resource. In the context of a carbon budget, however, the conversion of fallow forests to permanent agriculture is important because it reduces the amount of carbon stored on land. In the traditional form of shifting cultivation, the carbon released to the atmosphere on clearing is stored again during the re-establishment of forests in the fallow period. Over time, the net flux of carbon may be zero. If forests are eliminated permanently, however, the net flux is to the atmosphere.

The fraction of land cleared for agriculture that is permanent, as opposed to shifting, varies widely among regions: we estimate 66% for the tropics as a whole. This is a crude average which is based on estimates for individual countries and regions. Myers (unpublished data) estimates that the fraction of land cleared permanently may be as high as 95% in Brazil, and 90% in Columbia, Peru, Ecuador, and the Amazonian sector of Bolivia.

For all Latin America, however, an estimate of 85% was judged more appropriate because it accounts for the lower fraction of permanent conversions in Central America. In Asia, the fraction was estimated to be 70%, representative of a mean between high fractions where colonization of logged forests is common and low fractions where the more traditional shifting cultivation still predominates (for example, Papua New Guinea). In Africa, the overall average was estimated to be about 50%. It may be as low as 10% in Zaire but much higher in West Africa, especially since the drought in the Sahel has forced populations southwards into the forests. The fraction of clearing for subsistence agriculture estimated to result in the permanent clearing of forest was assumed to apply to the clearing of both fallow and non-fallow forests (Table 2).

The rates of temporary and permanent deforestation of non-fallow and fallow closed forests as derived from Myers' estimates¹⁰⁻¹² are shown in Table 2 and summarized in Fig. 1. Although Myers did not explicitly define these different pathways, the rates given in Table 2 and Fig. 1 are the only set of rates consistent with his data and with the fractions of temporary compared with permanent clearing given above. Deforestation of non-fallow closed forests (A in Fig. 1) was 7.0×10^6 hectare yr^{-1} . This rate includes the conversion of primary, secondary, and logged forests to both permanently cleared land (5.1×10^6 hectare) and to shifting cultivation (1.9×10^6 hectare yr^{-1}) (Table 2). The total is slightly lower than the one in Table 1 based on Myers' earlier estimates^{10,11}, and is close to the FAO/UNEP estimate of 7.3×10^6 hectare yr^{-1} . The breakdown between permanent and temporary deforestation differs from the FAO/UNEP estimate of 4.1×10^6 and 3.4×10^6 hectare of closed forest, respectively, but the difference between FAO/UNEP and Myers in this respect is not large.

The large difference is in their estimates of the change in fallow area. According to Myers' data 10.1×10^6 hectare of fallow forests are converted each year to permanently cleared land (Table 2). The net reduction in fallow (C-E in Fig. 1) is 8.2×10^6 hectare yr^{-1} , a reduction larger than the annual reduction in

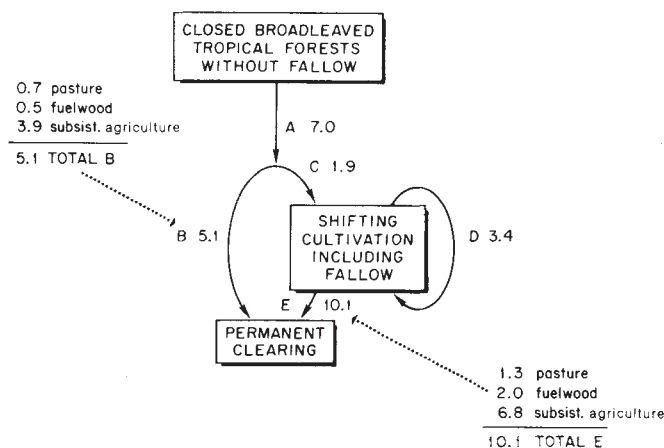


Fig. 1 Annual rates of conversion of tropical humid forests to other uses of land (10^6 hectare) based on data from refs 10–12, 14 and Myers (personal communication).

area of non-fallow forests. The FAO/UNEP study, in contrast, estimates that the area of fallow in closed forests is increasing annually at a rate of 3.4×10^6 hectare (ref. 9). If Myers is correct, a total of 15.2×10^6 hectare of forest land is being permanently cleared each year in the tropics, with 5.1×10^6 hectare coming from non-fallow forests and 10.1×10^6 hectare coming from fallow forests that were once part of the shifting cultivation cycle (Table 2, Fig. 1).

The second place where data from the FAO/UNEP survey contrast sharply with independent evidence is in growing stocks. Evidence from several reviews of terrestrial carbon stocks^{16–18} suggests that the estimates of growing stocks reported by FAO/UNEP are low by a factor of 2 when converted to carbon stocks (Table 3).

In this analysis, the volumes of growing stock reported country-by-country by FAO/UNEP were distributed among three types of forest in each region (America, Africa, and Asia) based on the density of growing stocks⁸. Mean volumes of wood, weighted by the area deforested⁸, were determined for forest type and region (Table 3). These mean volumes were converted to carbon with factors of 0.62 for the mean density of tropical wood¹⁹, 0.45 for the carbon content of wood, and 1.72 for those parts of the vegetation not included in commercial volume, such as twigs, leaves, roots, shrubs, and trees <10 cm in diameter²⁰. Similar conversion factors were derived independently by Brown and Lugo²¹.

The forests with high, medium, and low growing stocks in each region corresponded geographically to the evergreen equatorial forests, tropical seasonal forests, and tropical dry forests/woodlands, respectively, of Olson *et al.*¹⁸ Assignment of published estimates of biomass (cited in ref. 17) to these forest types on the basis of location yielded a second estimate of the carbon stocks for these forests. These estimates were about double those derived from the FAO/UNEP study. The same result was obtained by Brown and Lugo²¹ using more forest types.

The net release of carbon to the atmosphere as a result of clearing forests for permanent crops and pasture was calculated using a model described previously for global analyses^{2,22}. Logging and fuel-wood removals were incorporated in the analyses reported here by assigning masses of carbon simulating wood products to pools that oxidized at rates of 1/1, 1/10, or 1/100 yr^{-1} . This approach is consistent with the evidence that wood removals are most often coincident with deforestation. In Asia and Africa, logged forests are frequently settled by subsistence agriculturalists^{9–11}; in tropical America logging is generally carried out in forests destined for large development projects⁹. The model was expanded in this study to include the practice of shifting cultivation. Figure 2 shows the changes in carbon stocks that accompany shifting cultivation in the moist tropical forests of Asia (Table 3).

The model was used to calculate the net flux of carbon from

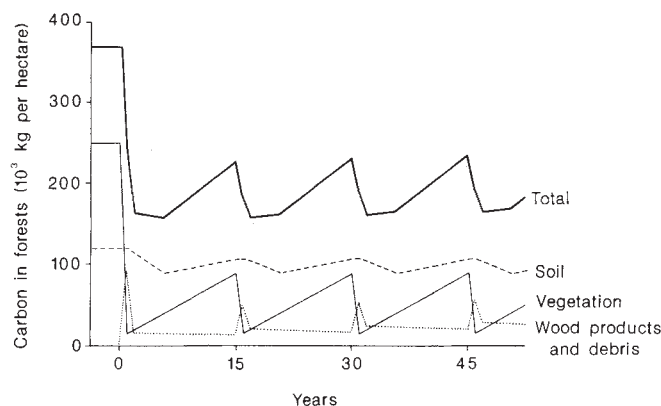


Fig. 2 Changes in the carbon content of vegetation, soils and pools of dead wood during three cycles of shifting cultivation.

tropical forests according to the three estimates of deforestation and the two estimates of carbon stocks described above. The carbon stocks of vegetation at the end of the fallow cycle (mature fallow) were determined from several sources^{23–25} as were the values for the carbon content of soils^{17,26,27} (Table 3). The conversion of forests to croplands and pasture was assumed to reduce the carbon content of the forest soils by 30% and 20%, respectively^{20,26}. Analyses were made for the three regions separately.

To allow a sufficient time for cohorts of the shifting cultivation model to stabilize, all modelling analyses were started in 1940. Because the only rates of deforestation given in the FAO/UNEP survey were average rates over the 5-yr period 1976–80, different assumptions about the rates of deforestation before 1980 were examined. Such differences affected the estimated flux of carbon in 1980 by less than 10%.

The net storage of carbon in forest plantations was not calculated in this analysis but is not thought to change the net flux significantly. For the tropics as a whole, the ratio of annual planting to deforestation is about 1:10 (ref. 9). In South-east Asia where the ratio is higher, the storage of carbon due to afforestation was $<5\%$ of the total net flux²⁰.

The net flux of carbon to the atmosphere in 1980 based on the FAO/UNEP estimate of deforestation of non-fallow forests in the tropics was about 1.4×10^{15} g C with the higher estimates of carbon stocks and 0.7×10^{15} g C with the lower estimates (Table 4). The three different estimates of deforestation gave remarkably similar estimates of flux for the tropics as a whole, reflecting similarities in the rates of deforestation for the entire tropics (Table 1). Within regions, however, different estimates of deforestation generated net fluxes that varied by about a factor of two. Summing the lowest and highest estimates of flux for each region gave ranges of 0.507 – 0.826×10^{15} g C, respectively, for the lower estimates of carbon stocks and 0.982 to 1.675×10^{15} g C for the higher estimates (Table 4).

The permanent deforestation of 10.1×10^6 hectare of fallow forests (Table 2, Fig. 1) was estimated to contribute another 0.4 to 0.8×10^{15} g C to the atmosphere in 1980 depending on the stocks of carbon assumed for fallow lands. For computation of

Table 4 Calculated net release of carbon to the atmosphere in 1980 (10^{15} g) from deforestation of non-fallow forests in the tropics (Table 1)

	FAO/UNEP	Myers	FAO
America	0.344 (0.649)	0.220 (0.400)	0.419 (0.783)
Africa	0.175 (0.390)	0.184 (0.406)	0.177 (0.394)
Asia	0.198 (0.362)	0.223 (0.486)	0.112 (0.190)
All tropics	0.717 (1.401)	0.627 (1.292)	0.708 (1.367)

Values in parentheses are based on calculations with higher stocks of carbon¹⁷. Other values are based on FAO/UNEP estimates of wood volumes converted to carbon stocks as described in text.

the loss of carbon associated with the conversion of fallow forest to permanent agriculture, the carbon content of the median aged cohort in the fallow cycle, rather than that of mature fallow, was used (Fig. 2). The resolution of the data did not warrant regional analyses, and the calculation was made for the tropics as a whole. The total net release of carbon from the tropics due to deforestation was thus estimated at between 0.9 and 2.5×10^{15} g C. Uncertainties in the rate of deforestation, in the stature of forests cleared, and in the length of time forests remain cleared contribute about equally to this range.

Both the range and the magnitude of the net flux of carbon in 1980 were lower in this analysis than previously reported by Houghton *et al.*² (1.3 – 4.2×10^{15} g C). Reduction of the upper end of the range resulted from a reinterpretation of Myers' estimate of deforestation. Houghton *et al.* assumed that two-thirds of Myers^{10,11} estimate for total conversion (22.5×10^6 hectare yr^{-1}) represented permanent deforestation, as Lanly and Gillis²⁸ had found in Latin American forests. As the analysis presented here reveals, the permanent conversion of non-fallow forests to other uses of land was only 7.0 – 7.5×10^6 hectare yr^{-1} in 1980 (Tables 1 and 2).

Data from the Production Yearbooks of the FAO were also used in a different way in this study from the earlier one. Houghton *et al.*² used changes in agricultural and grazing land to derive rates of deforestation. They distributed the total increase in agricultural area between both forest and non-forest systems, so that the decrease in area of forests was less than the increase in agricultural land. However, the loss of forests and woodlands has exceeded the growth of agricultural and grazing lands, according to the Production Yearbooks, and in the analysis presented here deforestation was determined from change in the area of forests (Table 1). The excess loss of forests has been to 'other land', a fourth category given by the Production Yearbooks. 'Other land' includes many types of land, the most important of which in this context is land that has lost its capacity to support either agriculture or forest. The carbon stocks of such lands are low.

The third analysis of Houghton *et al.*² based on population growth and per capita use of agricultural land gave a flux of carbon from the tropics in 1980 (2.1×10^{15} g C) within the range presented here despite a lower rate of deforestation. The net flux of carbon was lower in the present work for several reasons. The reduction of soil carbon following clearing of forests was 30% in this analysis (Table 3); in the earlier analysis it was 50%. The lower estimate was based on a more extensive review of literature^{20,26}. The estimates of the stocks of carbon in vegetation were also lower for Latin America and Africa. The use of carbon stocks derived from the FAO/UNEP study reduced these estimates still more. Finally, the harvest of wood in this analysis was coupled with the clearing of forests for agriculture^{9,10} and, therefore, accounted for a smaller net release of carbon than in the earlier analysis where harvest was treated independently of deforestation.

The range reported here (0.9 – 2.5×10^{15} g) is higher than the ranges found in recent studies by Detwiler *et al.* (personal communication) and Molofsky *et al.*³, 0.5 – 1.8×10^{15} g C and 0.6 – 1.1×10^{15} g C, respectively. Both studies were based on models similar to the one used here and both used the same FAO/UNEP study to provide one estimate of forest biomass and one estimate of deforestation. If the deforestation of fallow lands is ignored in the analysis presented here, the ranges found by all three studies are similar.

The agreement, however, is more a reflection of the data available and the similarity of methods of analysis than a measure of how accurately the rates of deforestation or the carbon stocks of vegetation and soil are known. If the estimates of deforestation shown in Table 1 (non-fallow forests only) are assumed to represent the best estimates, agreement seems to be within about 100%, although in Asia, and India in particular, there are differences greater than 100%.

The major discrepancy between the estimates of Myers and FAO/UNEP is the direction and rate of change in area of fallow.

The size of the discrepancy is dependent on the fraction of deforestation estimated by Myers to be permanent. Lower fractions would reduce the difference and reduce the release of 0.4 – 0.8×10^{15} g C yr^{-1} calculated to result from the elimination of fallow forests. Resolution of the difference probably requires the use of extant and future data from satellites. Monitoring tropical forests with Landsat can establish rates of deforestation and whether the deforestation is permanent or temporary^{7,29}. Landsat can also be used to establish the age, and indirectly the carbon stocks, of fallow forests regrowing on lands cleared since 1972.

The difference between estimates of carbon stocks based on measurements of biomass¹⁷ and those derived from estimates of wood volumes reported by FAO/UNEP^{8,21} (Table 3) affects the range of flux estimates about as much as the uncertainty of deforestation rates. It is not clear which of the two estimates is based on samples more representative of extant forests, and whether the lower stocks of carbon are a result of natural variation or a result of disturbance by man.

The latter distinction is important because analyses here and elsewhere^{2–6} use change in the area of forests to calculate changes in the amount of carbon stored on land. Reduction of carbon stocks within areas of forest have been ignored, largely because the process of degradation is gradual and not as readily documented as deforestation. Such degradation occurs throughout the tropics, however, as a result of logging, harvest of fuelwood, deliberate burning, and grazing^{8,9}. The extent of this degradation and the net release of carbon associated with it cannot be evaluated at present; the reduction per unit area varies considerably and the areas affected are large. The direction of the net flux of carbon is clear, however. It is from land to the atmosphere. Thus the net releases of carbon from biota and soils estimated here are probably underestimates.

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Entrainment and vertical transport of deep-ocean water by buoyant hydrothermal plumes

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Submarine hydrothermal vents produce effluent plumes in the water column which can be easily detected with tracers such as ^3He , manganese and methane¹⁻³. Comparison of the tracer concentrations and temperature anomalies in such plumes with direct measurements of the pure vent waters shows that the plumes are typically mixtures of 1 part vent water with 10^4 parts ambient sea water¹. However, the extent to which hydrothermal plumes entrain and transport sea water and contribute to deep-ocean mixing is not known. We present here hydrographic data collected in the vicinity of an active hydrothermal field on the Endeavour segment of the Juan de Fuca Ridge where a well-defined effluent layer resides in the water column ~200 m above the depth of the vent field. The temperature and salinity signature of this layer indicates that a small volume of hot vent water can be very efficient at entraining ambient sea water. Directly above the Endeavour vent field, the effluent layer is composed of 0.01% vent water, 30% ambient water normally found at that depth, and ~70% entrained water which has been transported from deeper in the water column.

As shown in Fig. 1, the Endeavour segment of the Juan de Fuca Ridge is a 170-km section of mid-ocean spreading centre bounded by the Cobb Offset in the south and the Sovanco Fracture Zone in the north. In 1984, two *Alvin* submersible expeditions to this area discovered and mapped an active hydrothermal system within the axial valley of the spreading centre^{4,5}. Although the exact extent of the hydrothermal zone is unknown, a large vent field at 2,200 m depth near 47°57' N, 129°06' W was mapped in detail by *Alvin*. The field covers an area 150 × 250 m and contains many 'black smokers' venting water at temperatures up to 400 °C (refs 5, 6). The hydrographic properties in the region were mapped by vertical profiling with a Neil Brown conductivity-temperature-depth (CTD) probe, and for near-field studies the CTD was mounted directly on the submersible during several dives⁷.

The effluent layer over the Endeavour segment was discovered with the striking profiles shown in Fig. 2, which were collected in 1983, 1 yr before the existence of hydrothermal venting was

confirmed by *Alvin*. Figure 2 shows CTD profiles of temperature, salinity and potential density at TT-175, Station 6, ~4.4 km east of the vent field. For comparison, a 'background' temperature profile is shown for TT-175, Station 1, ~26 km east of the vent field. Relative to this background profile, the Station 6 profile shows a clear temperature excess of ~0.050 °C over the interval 1,960–2,140 m depth. This feature is present to some degree in all the CTD profiles collected within 5–10 km of the vent area both in 1983 and 1984, and we conclude, therefore, that this hydrothermal layer is a long-lived feature of the water column in this region. The salinity and density profiles also reveal the existence of the effluent layer: the top of the layer coincides with a pronounced hump in the salinity, and the variation of density with depth within the layer is much smaller than above or below, indicating lower stability (lower Brunt-Vaisala frequency). Note that despite the dramatic temperature excursion, the layer is nonetheless stable, that is, the water in the layer has no tendency to rise or descend.

The distinguishing features of this layer are even more apparent in property-property plots of potential temperature, salinity and potential density as shown in Fig. 3. In the north-east Pacific, these three properties form mixing lines which are almost perfectly linear for water masses sampled below ~1,000 m depth. The injection of hydrothermal vent water is one process which violates this linear mixing relationship, and the anomalous properties of the layer are exhibited as clear deviations from the linear trend. We make the reasonable assumption that the density of the layer is nearly identical to that of the water which would reside at the same depth in the absence of hydrothermal activity. Figure 3 indicates, therefore, that the layer has an excess of temperature of ~0.050 °C, and excess salinity of ~0.007‰, relative to the predicted properties for water at that density. The excess temperature and salinity of the layer exactly compensate each other to produce a mixture of the appropriate density.

The excess temperature of the layer has obviously come from the hot vent water, and suggests that vent water averaging 300 °C has been diluted by a factor of ~7,000 before reaching the layer. However, the salinity excess cannot be attributed directly to the vent water, as the salinity of the vent water would have to be >60‰ to produce the salt anomaly in the effluent layer. The pure vent water samples collected by *Alvin* at this site have salinities quite close to that of ambient sea water⁸, and CTD data collected by *Alvin* in near-field buoyant plumes show no evidence of salinities higher than ambient (see Fig. 4). Therefore, we attribute the salinity excess in the effluent layer to the entrained water which has been transported vertically by ~200 m in the water column from a region of higher ambient salinity. Using the salinity difference between bottom water in the vicinity of the vent field (2,200-m depth) and water at the depth of the layer (2,000 m), and assuming all of the entrainment occurred

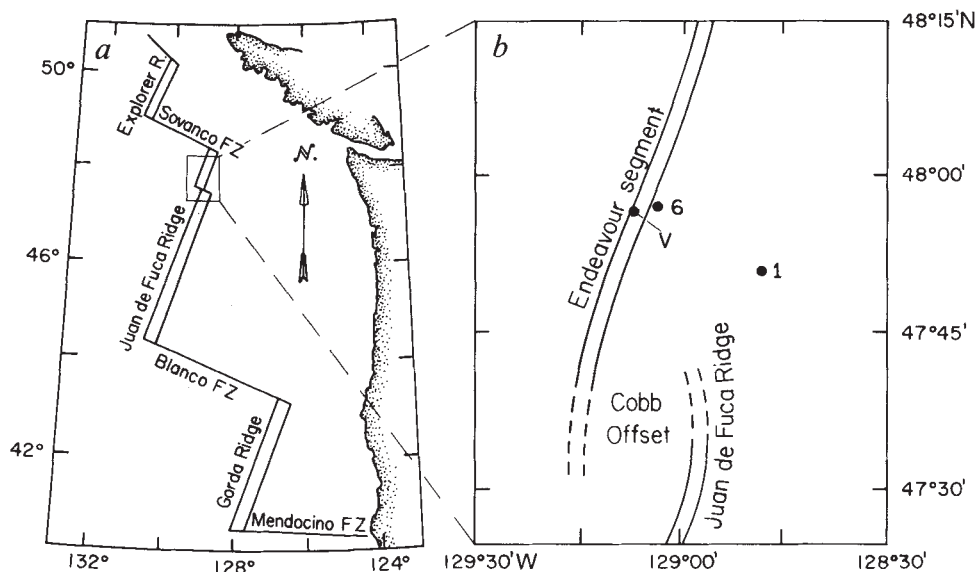


Fig. 1 a, Map of active spreading zones in the north-east Pacific. b, Detailed map of study area on Endeavour segment of the Juan de Fuca Ridge, showing the location of the hydrothermal vent field (V), and the hydrographic stations (1 and 6) occupied during expedition TT-175 in 1983.

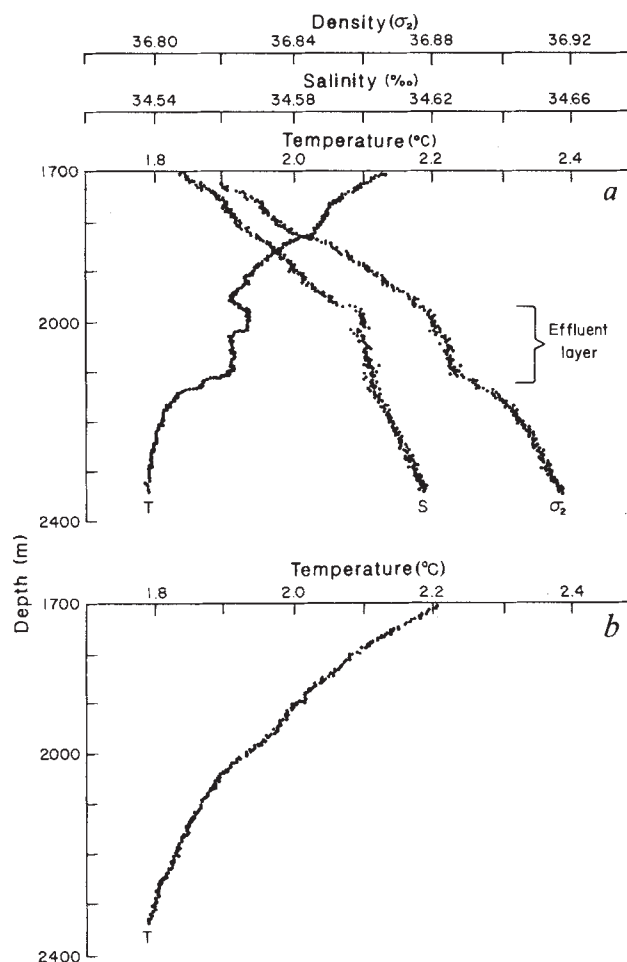


Fig. 2 a, Vertical profiles of temperature, salinity and potential density for Station 6, expedition TT-175, collected with a Neil Brown CTD in August 1983, at 47°57.5' N, 129°03.0' W, ~4.4 km east of the Endeavour hydrothermal vent field (see Fig. 1). Only the depth interval from 1,700 to 2,400 m is displayed. Potential density is expressed as σ_2 in units of $\%$ referenced to 2,000-dbar pressure. Brackets indicate the hydrothermal effluent layer which lies between 1,960 and 2,140 m depth. b, CTD temperature profile for Station 1, expedition TT-175 collected in August 1983, at 47°51.8' N, 128°47.7' W, ~26 km east of the hydrothermal vent field (Fig. 1). This serves as a 'background' profile, as it is largely unaffected by hydrothermal input.

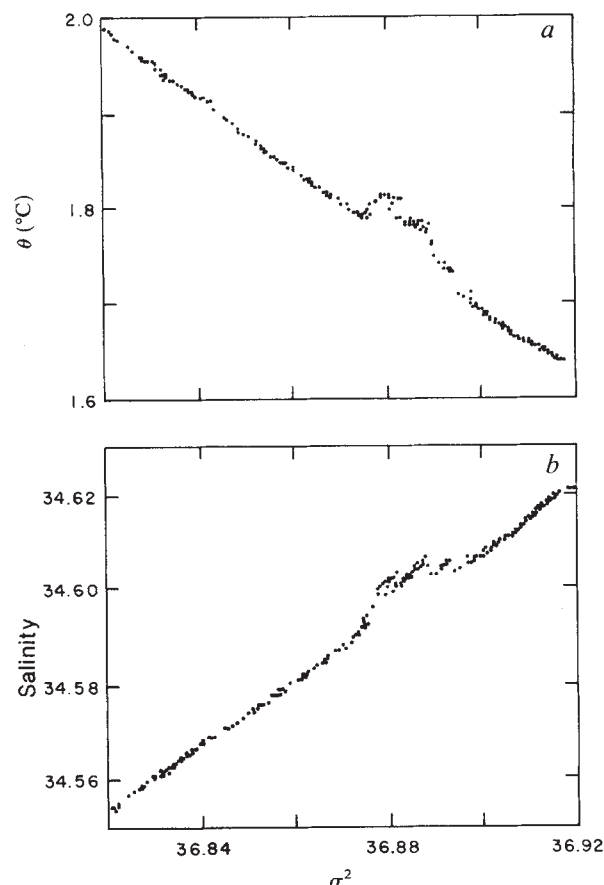


Fig. 3 Property-property plots of: a, potential temperature (θ) versus potential density (σ_2); and b, salinity versus potential density, for Station 6, expedition TT-175. The effluent layer (see Fig. 2) produces a marked excursion from the linear mixing trend which holds for ambient water masses unaffected by hydrothermal input.

at 2,200 m depth, we calculate that the layer must consist of 70% entrained water and only 30% water which would normally reside at 2,000 m depth. If we make the more realistic assumption that entrainment has occurred throughout the depth interval from 2,200 up to 2,000 m, then the proportion of entrained water in the layer is even higher.

Note that this entrained component cannot be detected with vent water tracers such as ^3He , and heat. Only a property such as salinity, which varies appreciably with depth in ambient sea water and has no signal in the vent fluids, can be used to estimate the extent of hydrothermal entrainment.

In addition to the stable effluent layer centred at 2,000 m depth, we were able to sample several unstable buoyant plumes with the CTD mounted on *Alvin*. One example is the ascent profile for dive 1451, shown in Fig. 4, in which the submarine passed through a plume ~0.10 °C above ambient shortly after leaving the Endeavour vent field. The potential density within this plume is lower than the overlying water, indicating that this is a buoyant water mass which is in the process of rising and entraining ambient water.

Fluid dynamical models of buoyant plumes in a stable stratified environment make predictions which can be compared with our observations. Using a buoyancy flux of $1.7 \times 10^7 \text{ cm}^4 \text{ s}^{-3}$ estimated for a single black smoker, and an ambient density

gradient of $1.46 \times 10^{-6} \text{ s}^{-2}$ (Fig. 2a), the numerical model summarized by Turner⁹ predicts a maximum plume height of ~370 m and a ratio of entrained water to vent water of 3.4×10^4 at the top of the plume. Thus the model agrees roughly with our observations, which indicate a 200 m plume height and an entrainment ratio of $\sim 10^4$. The discrepancy suggests that although black smokers are the most visible type of discharge, they may not be the most typical. Thus a large fraction of the effluent layer may be a result of diffuse hydrothermal flows which individually produce buoyancy fluxes much smaller than that of a black smoker.

Stommel¹⁰ has calculated that the heat flux from hydrothermal vents should drive zonal circulation cells in the deep ocean that extend westwards from north-south trending ridges such as the East Pacific Rise and the Juan de Fuca Ridge. While Stommel assumed a plume entrainment factor of 10^3 and a plume temperature of 0.3 °C, our results indicate 10 times lower plume temperatures and 10 times more entrainment. We calculate that each calorie of hydrothermal heat should result in the vertical transport of ~25 cm³ of ambient water. A typical smoker contributes $\sim 10^7 \text{ cal s}^{-1}$, which is roughly equal to the average theoretical hydrothermal heat flux from a 1-km-long ridge segment spreading at 3 cm yr⁻¹ half rate^{11,12}. The 170-km-long Endeavour segment should introduce $2 \times 10^9 \text{ cal s}^{-1}$ and produce ~0.05 sverdrups ($5 \times 10^4 \text{ m}^3 \text{ s}^{-1}$) of vertical transport when the episodic hydrothermal activity is averaged over geological time scales. Thus the ~40 black smokers that are active at the Endeavour hydrothermal field are supplying a substantial fraction of the average hydrothermal heat flux for the 170-km long ridge segment.

The Endeavour vent field is only one of five or six active geothermal zones on the Juan de Fuca/Explorer ridge sys-

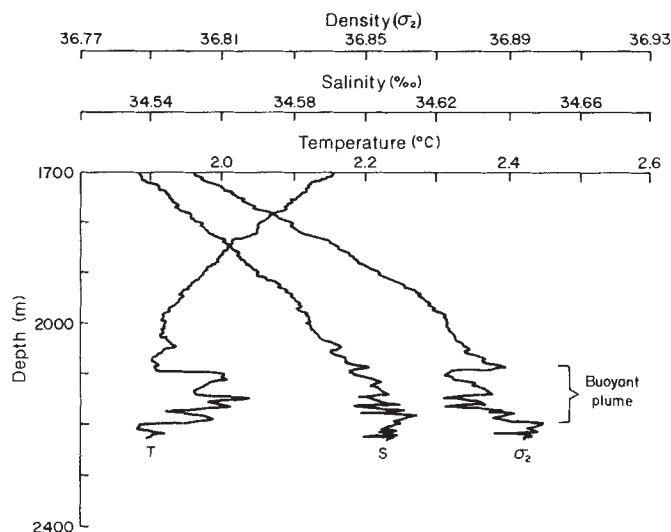


Fig. 4 Vertical profiles of temperature, salinity and potential density collected in September 1984, at the Endeavour hydrothermal vent field (see Fig. 1). The data were collected during the ascent of dive 1451 with the CTD mounted on the submersible *Alvin*. Shortly after leaving the bottom, the submarine passed through a hydrothermal plume $\sim 0.1^\circ\text{C}$ above the ambient temperature. The potential density signature shows that this was an unstable buoyant plume, quite different from the stable effluent layer shown in Figs 2 and 3.

tem^{4,5,13-17}. Applying our average hydrothermal heat flux calculation on a broader scale, the $\sim 760\text{-km}$ -long spreading system should produce ~ 0.2 sverdrups of vertical 'pumping' which, due to the considerable topography of the ridge crest, would deposit water onto several different density horizons. This transport is significant compared with the abyssal flows predicted for the deep north Pacific^{18,19}, suggesting that hydrothermal activity may influence the deep circulation patterns for a considerable distance from the Juan de Fuca Ridge axis, as predicted by Stommel¹⁰.

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Absolute motion and evolution of the Bouvet triple junction

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The absolute motion of the Bouvet triple junction has an important role in its mechanical evolution. At present, the relative velocity vectors of the South American, African and Antarctic plates form an obtuse isosceles triangle, and both the ridge-ridge-ridge (RRR) and ridge-transform-transform (RFF) configurations of the Bouvet are potentially stable. Sclater¹ demonstrated that the Bouvet twice alternated between RRR and RFF in the past 20 Myr. The reasons for these changes are unclear, but the analysis of the absolute velocities of the two configurations presented here provides some insight. The motion of the RRR configuration of the Bouvet, with respect to a hotspot reference frame, carries the junction to the north-west (343°) at 14.7 km Myr^{-1} , whereas the RFF form moves to the south-east (157°) at only 5.8 km Myr^{-1} . The RFF configuration is stable only when the relative velocity triangle is precisely isosceles. As the RFF junction migrates away from the isosceles line, it becomes increasingly unstable, until it is forced to switch to RRR. The RRR junction then drifts back to the isosceles line where it reverts to RFF.

The classic notion of triple junction stability introduced by McKenzie and Morgan² is based on constant plate velocities. McKenzie and Parker³, however, recognized that changes in relative velocities, which necessarily accompany the motion of a triple junction on the surface of the Earth, will affect its stability.

Minster and Jordan's AM1-2 absolute poles of rotation⁴ for the South American (SOAM), African (ARFC), and Antarctic (ANTA) plates, were utilized to calculate absolute plate velocities and to locate the locus of isosceles velocity triangles (Fig. 1) in the vicinity of the present Bouvet triple junction.

The actual Bouvet triple junction falls some 6° south of the calculated 'isosceles line'. This discrepancy probably reflects uncertainties in the absolute poles of the AM1-2 model because Sclater has shown that the relative velocities at the Bouvet junction form an isosceles triangle within observational error¹. To be consistent with the AM1-2 velocities, we assume here that the Bouvet triple junction is at 61.8°S ; 0°E .

Figure 2a is a velocity space diagram for the Bouvet triple junction illustrating both RRR and RFF configurations. The

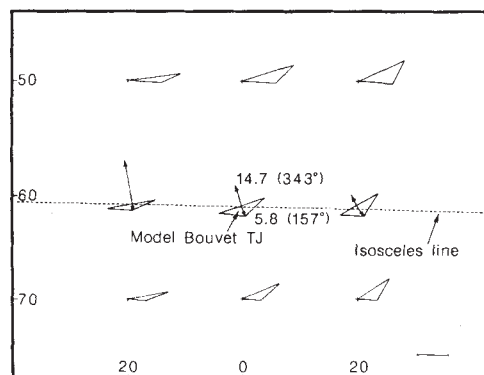


Fig. 1 The position and orientation of the isosceles line in the South Atlantic calculated from Minster and Jordan's AM1-2 absolute motion model¹. The velocity of the triple junction in RRR and RFF modes is 14.7 km Myr^{-1} at 343° and 5.8 km Myr^{-1} at 157° , respectively. In RFF mode, the junction migrates south-east away from the isosceles line becoming increasingly unstable as the velocity triangle becomes distorted. Scale bar, 20 km Myr^{-1} .

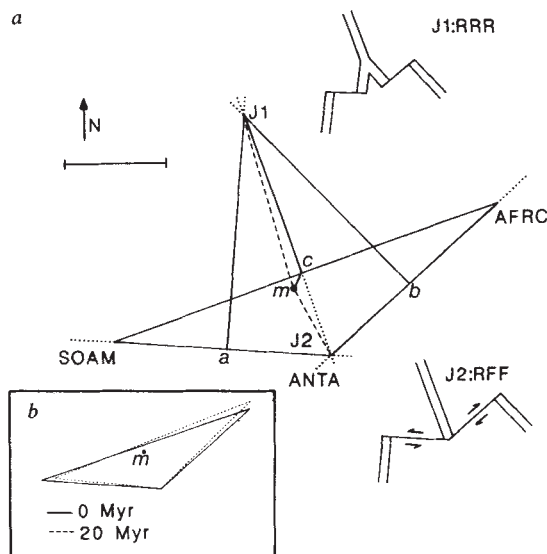


Fig. 2 *a*, A velocity-space diagram for the Bouvet triple junction with both RRR and RFF modes superimposed. In RRR mode vector, a -J1 and b -J1 are the growth rates of the SOAM-ANTA, AFRC-ANTA ridges and vector c -J1 is the rate that the SOAM-AFRC ridge is consumed. In RFF mode, vectors a -J2 and b -J2 are the rates of growth for the respective transforms, and vector c -J2 is the rate of ridge growth. m is the location of the mantle reference frame. Vectors m -J1 and m -J2 are velocities of the triple junctions in RRR and RFF modes, respectively. Vector mc is the absolute velocity of the Mid-Atlantic Ridge. Two insets illustrate the direct space configurations. *b*, Changes in the shape of the relative velocity triangle which accompany the migration of a RFF junction away from the isosceles line. The rotation of the ANTA-AFRC and ANTA-SOAM vectors are much exaggerated to illustrate the fact that the original transforms become 'leaky'. Scale bar, 8 km Myr^{-1} .

RFF junction is stable only when the relative velocity triangle is isosceles. The vectors a -J2 and b -J2 are the rates of growth of the paired transforms which form when the junction is RFF. In the RRR mode, vectors a -J1 and b -J1 represent the growth rates of the SOAM-ANTA, AFRC-ANTA ridges. Vector c -J1 is the rate that the SOAM-AFRC ridge is consumed. The absolute velocities of the RRR and RFF junctions are vectors m -J1 and m -J2 respectively; m denotes the velocity of a mantle reference frame. Triple junction velocity vectors along the isosceles line are shown in Fig. 1. The Bouvet triple junction in

RRR mode has an absolute velocity of 14.7 km Myr^{-1} at 343° . When RFF, the junction migrates at 5.8 km yr^{-1} towards 157° . The orientation of these vectors change systematically along the isosceles line although they are oriented consistently in directions opposite one another.

Both the Conrad and Bouvet fracture zones are $\sim 150 \text{ km}$ in length¹. Assuming that the absolute velocities of the three plates have not changed significantly during the past 30 Myr, their rate of growth would have been 8.1 km Myr^{-1} (vectors a -J2 and b -J2, Fig. 2). This would require that the Bouvet junction remained in the RFF mode for 18.5 Myr. Using this and other information derived from the AM1-2 model, we can infer the following history for the Bouvet junction from the inception of the paired Bouvet and Conrad fracture zones.

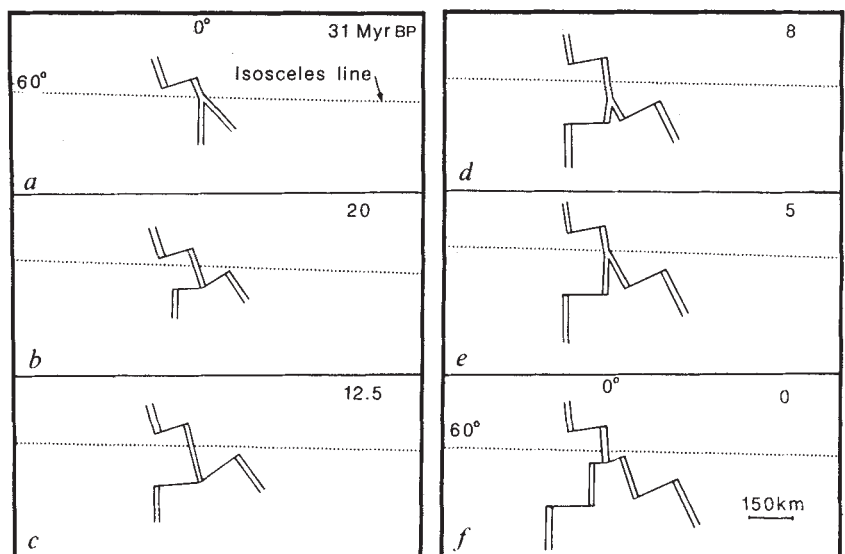
Sometime before 31 Myr BP, a RRR version of the Bouvet junction existed south of its present location. Its absolute velocity carried it northwards at 14.7 km Myr^{-1} . At 31 Myr BP the junction intersected the isosceles line and changed to RFF mode (Fig. 3*a*). In RFF mode (Fig. 3*b*), the junction migrated with a velocity of 5.8 km Myr^{-1} at 157° away from the isosceles line until 12.5 Myr BP when it reverted to its old RRR configuration (Fig. 3*c*). During the RFF episode, the SOAM-AFRC ridge grew 120 km and both the Conrad and Bouvet transforms were generated. As RRR, the junction then drifted back towards the north-west (343°) consuming the SOAM-AFRC ridge and lengthening the SOAM-ANTA, AFRC-ANTA ridges in the process (Fig. 3*d*). Finally, 5 Myr ago, the junction arrived in the vicinity of the isosceles line (Fig. 3*e*) and switched back to RFF. Since then, the junction has been moving south-east away from the isosceles line in its present RFF configuration (Fig. 3*f*).

The inferred sequence of events is similar to the history of the junction deduced by Sclater *et al.*, from magnetic data¹. The timing of the configuration changes are, however, slightly different. Magnetics date the first RRR to RFF transition at 10 Myr BP and the second at 5 Myr BP. These discrepancies reflect either the inaccuracy of the absolute motion model or suggest that the plate motions have changed slightly during the past 30 Myr. Nonetheless, the overall agreement supports the idea that the minor changes in relative velocities which accompany junction migration can have a significant role in its evolution.

Several questions remain. For example, the RFF configuration of the Bouvet junction should be stable only on the isosceles line. Its migration away from the isosceles line would render it immediately unstable. Yet it seems to have survived for at least 18.5 Myr (31–12.5 Myr BP) in RFF mode. Why?

The reasons behind its persistence may lie in the nature of the changes in plate velocities which accompany its south-east migration (Fig. 2*b*). The rotations of the relative velocities are

Fig. 3 A model of evolution for the Bouvet triple junction based on the AM1-2 absolute motion model¹ and the length of the Bouvet and Conrad fracture zones. At the isosceles line (*a*) (31 Myr BP) the junction switched from RRR to a more geometrically stable RFF mode. As it migrated southeastwards (*b*) (20 Myr BP) the junction generated the paired Conrad and Bouvet transforms and extended the length of the Mid-Atlantic Ridge. In *c* (12.5 Myr BP), the transforms reached their final length of 150 km, and the junction switched back to RRR mode and began migrating in a northwesterly direction. As RRR (*d*), the junction drifted back towards the isosceles line. In *e*, the junction intersected the isosceles line and began generating new paired transforms until the present (*f*).



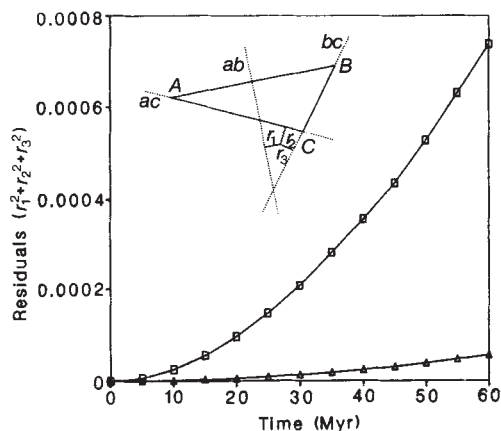


Fig. 4 Changes in the degree of geometric instability (residuals) as the RRR ($\square$) and RFF ($\triangle$) configuration of the Bouvet triple junction drifts away from the isosceles line. The orientations of the boundaries are assumed to be fixed, and spreading is assumed to be symmetrical. Instability arises because the relative velocities change with time. The RFF configuration is least affected by small changes in the velocity triangle, and it seems to be the preferred configuration in the vicinity of the isosceles line. The inset illustrates the geometric significance of the residuals.

such that the growing transforms develop a small component of extension. In other words, they become progressively 'leaky'. The actual rotations amount to only 1° over a period of 20 Myr, but this may be sufficient to make them mechanically feasible.

When the triple junction finally changes to RRR configuration, the newly generated slow spreading ridge segments must undergo rotation as the relative action vector changes during north-west migration. The MAR must also undergo a change in the spreading direction (Fig. 2b). This rotation is possibly accommodated by the generation of secondary transform faults along these ridge segments.

Questions also surround the reason why the junction switches from a RRR mode to RFF in the vicinity of the isosceles line. A partial answer may be suggested by the relative geometric instabilities of the two configurations. The location of the triple junction in two-dimensional velocity space is given by a set of three equations involving the three-plate velocities and boundary orientations. If these equations have a unique solution, the junction is stable in the sense of McKenzie and Morgan² and the velocity of the triple junction is well defined. If no solution exists, the triple junction is unstable. We suggest that the residuals of a least-squares fit in the case of an unstable junction can be used as a measure of the degree of geometric instability (see inset, Fig. 4).

For the Bouvet junction, the residuals for the RRR and RFF modes are plotted in Fig. 4 as a function of their travel time away from the isosceles line. In these calculations, the boundary orientations are fixed, and the spreading is considered to remain symmetric. The residuals increase for RRR and RFF modes as the junction migrates. However, the instability of the RFF junction builds up much more slowly than RRR configuration. In the vicinity of the isosceles line (the origin of Fig. 4), the RFF junction is less susceptible to changes in the relative velocities perhaps explaining why it is the preferred state.

We conclude that the Bouvet triple junction is trapped in the vicinity of an isosceles line in the South Atlantic. Along this locus, the junction can exist in either RRR or RFF configuration, although RFF seems to be more stable. As the absolute motion of the RFF junction carries it away from this locus, it eventually becomes mechanically unstable and switches to a RRR configuration. The RRR junction has an absolute motion which drives it back to the isosceles line where it reverts to its original RFF configuration. Because the orientations of the absolute triple junction trajectories straddle the isosceles line, the Bouvet junction is destined to alternate between episodes of RRR and RFF as long as the absolute plate velocities remain unchanged.

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Geometry of propagating continental rifts

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Reconstruction of past plate configurations through palinspastic restoration of continental margins¹⁻⁴ requires a detailed knowledge of the geometries of horizontal continental extension. Extension is often restricted to discrete, linear zones termed rifts^{5,6}, and rift models that invoke propagating vertical dykes and cracks⁷⁻¹⁰ are useful for assessing the relative significance and timing of uplift, subsidence and extension in the tectonic evolution of rift basins. However, many models do not adequately account for the observed asymmetry of rifts. I describe here the general three-dimensional character of young and aborted continental rifts, which can be used to derive a structural model for the propagation of rifts in continental lithosphere. The rifts become asymmetric as a consequence of the role played by low-angle normal faults in the overall rift geometry.

Continental rifts are typically a few tens of kilometres in width, and several tens to a few hundred kilometres in length (Figs 1, 2). That these rifts are in some cases precursors to the complete rupturing of continental lithospheric plates seems to

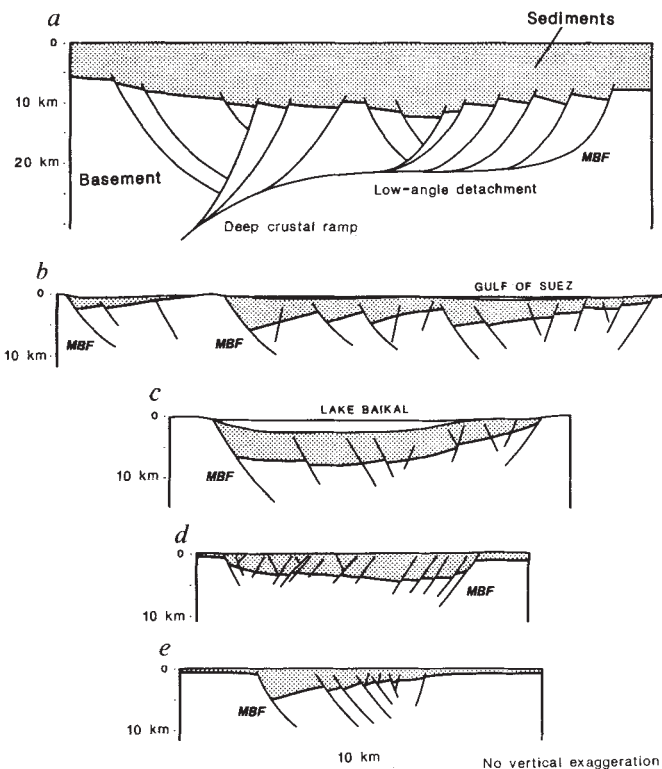


Fig. 1 Asymmetry of continental rifts in cross-section. Rifts commonly show half-graben-like forms in cross-sections taken normal to their long axes, with most basin relief generated by a single rift bounding fault (main bounding fault, MBF), or a system of a few main faults, which are inferred to bottom out to a low-angle detachment surface. *a*, Central Graben, North Sea, section (a submerged continental rift) is an approximate depth conversion of a time section presented by Gibbs²³. *b*, Section crossing southern Gulf of Suez, constructed from industry well and seismic data. *c*, Baikal Rift, sediment thicknesses from ref. 43, but internal faulting is largely schematic. *d*, Rhine Graben, from ref. 20. *e*, White Nile Rift, in part based on the gravity work of Browne *et al.*¹⁹.

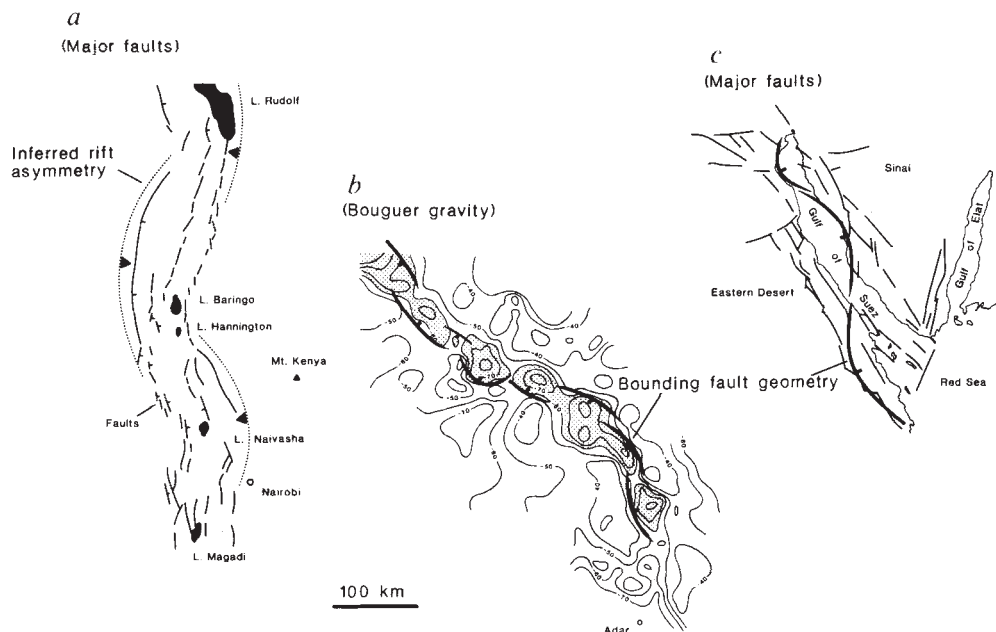


Fig. 2 Geometry of continental rifts in plan view. The major faults of continental rifts follow curvilinear patterns in map view, defining a sub-basin geometry that repeats generally every 50–150 km. Similar curvature is seen in the bounding faults of the Basin and Range Province⁴⁴. The asymmetry of the rifts in cross-section commonly reverses at each successive sub-basin, although not in every case. *a*, Gregory Rift, Kenya. Surface traces of major faults are from ref. 45. *b*, White Nile Rift, Sudan. Gravity data are from ref. 19. *c*, Suez Rift, Middle East. Surface faulting from ref. 17. Sub-basin geometry in the Gregory Rift (*a*) is more complex than portrayed here.

be well established by studies of the African-Arabian plate boundary (Red Sea and related rifts)^{11–15}. At the other extreme, continental extension may be distributed over broad zones, measuring hundreds of kilometres in each horizontal direction, as in the Basin and Range of the western United States. Whether the Basin and Range structure evolves from a few, initially discrete rifts, and whether it can eventually lead to continental breakup, are unanswered fundamental questions of continental tectonics.

Continental rifts are strongly asymmetrical in cross-sections normal to their long axis^{12,16} (Fig. 1). This asymmetry is evident in gravity-field gradients^{17–19}, relative rift shoulder elevations, sediment isopach maps^{20,21} and seismic reflection profiles^{22,23}. Meinesz²⁴ suggested that rifts would develop an initial asymmetry corresponding to the first formed bounding fault. As crustal flexure continued, the opposing fault breaks to produce a symmetrical graben form. Most models of rift formation^{8,12,25–30} have envisioned continental rifting, and hence the development of continental margins^{1,2,31} as essentially a symmetrical process at the scale of the lithospheric plate.

Many workers have suggested that the main faults of rifts pass completely through the crust to a zone of structural decoupling^{27,32,33} (Fig. 3). Recent geological and geophysical studies indicate that most of the extension within rifts and the larger Basin and Range framework is accommodated through displacement on low-angle normal faults^{22,23,24,29}. If the bounding faults of rifts link into such structures, and these fault systems cut entirely through the crust or lithosphere³⁴, then an asymmetrical rift structure would be expected. A symmetrical

arrangement of opposing low-angle detachments is untenable, as movement on one would offset the other, probably leading to the locking of one of the detachments.

Several geometries have been proposed for the detachments that are thought to underlie rifts. Wernicke³⁴ has suggested that these faults or fault zones are essentially planar on a regional scale. Alternatively, they may bottom out at a major crustal discontinuity such as the brittle-ductile transition³¹, and then plunge downwards at deep crustal ramps²³. Similarly, great diversity is observed in the internal fault patterns of rifts. Both planar fault arrays and curved (listric) fans are encountered in continental rifts^{35,36}. Differential rotation across larger rift faults, however, commonly indicates that they define a listric system. Listric faults curve in plan view as well as cross-section, but only towards the hanging wall block. Curvature towards the footwall is not kinematically viable. This effectively limits the horizontal extent of a single rift bounding fault (or system of parallel faults) to a few tens of kilometres (Fig. 2). Detailed seismic surveys reveal that rifts are typically broken into structurally-coherent compartments by transverse features^{31,37} analogous to lateral and oblique ramps in contractional tectonic settings. These structures referred to as 'transfer faults'²³, are a syn-rift feature, although they may inherit a pre-existing structural grain.

On a larger scale, rifts also break up into sub-basins, which may form as isolated depositional systems (Fig. 2). Sub-basin boundaries may be defined by major transfer faults, pre-existing crustal discontinuities, or in some cases by younger strike-slip faults which simply offset an older rift. Significantly, rift asym-

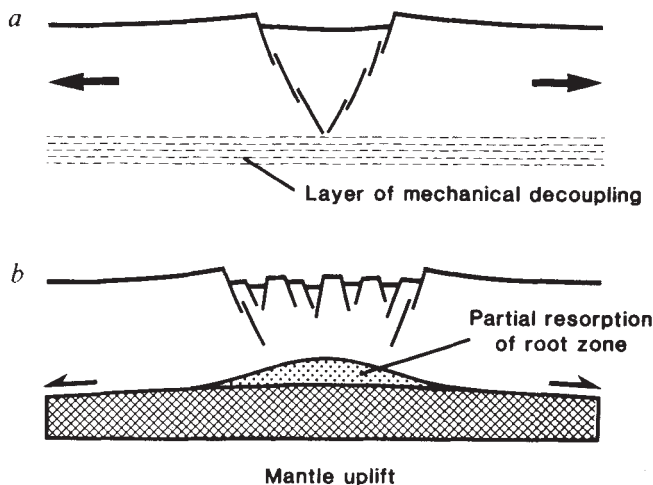
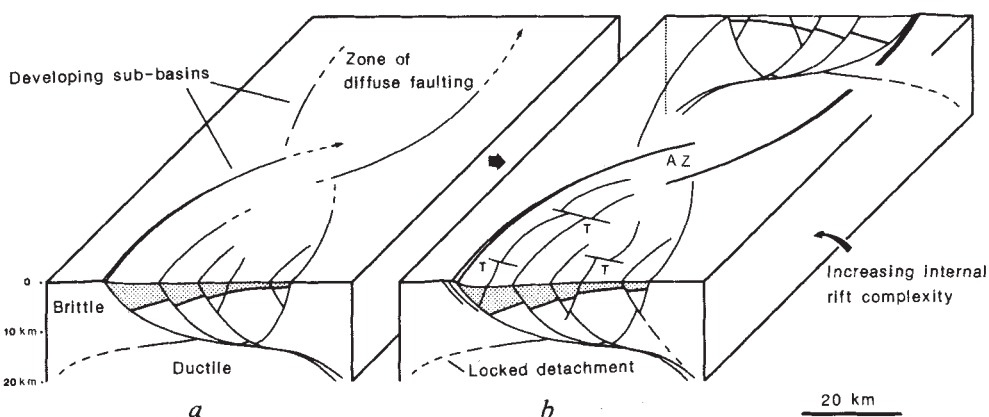


Fig. 3 Classical rift model of a brittle upper crust deforming above a ductile zone of mechanical decoupling (after ref. 30). The principal structural form of the rift itself is that of a symmetrical graben, defining a triangular wedge-block at depth.

Fig. 4 Proposed model for the propagation of continental rifts. Upper crustal extension may initiate as a broad zone of diffuse faulting (a), but quickly evolves to a system composed of a few main listric faults (and perhaps in some cases planar fault-bounded blocks³⁵) above two oppositely-directed detachments. For both detachments to remain operative, their deeper sections would need to be repeatedly recut, due to their mutually offsetting geometry. This rarely occurs, judging from the observed asymmetry of most continental rifts (Fig. 1), and one detachment locks. The active detachment propagates along the rift axis (towards the pole of opening), but curves inwards to form a large-scale scoop-like structure⁴⁴. A theoretical treatment of this fault propagation would have to consider how this entire structure evolves—both the growth of the near-surface high-angle faults and the lateral propagation of the shallow-dipping detachments. Eventually the curving, active listric system departs enough from the overall rift trend to favour a new detachment system, which links to the old at a complex area referred to by Derksen and others³⁹ as an 'accommodation zone' (AZ). Again, opposing detachments may initially form, and the advantage may go to the detachment of opposite polarity. In this case, a reversal of rift asymmetry occurs, with greater down-faulting and sedimentation adjacent to the new main bounding fault (b). Detachment systems may overlap or merge at accommodation zones in a variety of configurations, but in the plan view shown here, a cross-section normal to the rift axis at the accommodation zone would actually appear to be a symmetric graben in form³⁹. The important cross-faults in rifts referred to by Gibbs²³ as 'transfer faults' (T) are kinematically similar to accommodation zones, on a smaller scale.



metry frequently reverses at sub-basin boundaries, as was early documented for the Gulf of Suez Rift³⁸ (Fig. 2). The change in asymmetry between adjacent rift sub-basins strongly suggests that the underlying low-angle detachment system also changes polarity. The area of asymmetry reversal was first termed a "hinge zone" by Moustafa³⁸ for the associated regional dip reversal on rotated fault blocks. As asymmetry remains constant at some sub-basin boundaries, with only an offset in the rift bounding fault (Fig. 2), these major crustal structures have been more appropriately referred to as "accommodation zones"³⁹.

Young rifts, and those that failed at an early stage of continental extension, can then be thought of as large-scale half graben (mega-half graben of Cohen⁴⁰), which flip more or less regularly along the axis of the rift (Figs 1, 2). When rifting is first initiated, two opposing low-angle detachments may develop simultaneously, and propagate in the direction of the pole of opening for the two diverging continental masses (Fig. 4). One of the detachment systems soon locks, and the half-graben form develops. At some point along the incipient rift zone, the opposing detachment gains the initial advantage, and a reversal of asymmetry is forced on the bounding fault system. There appears to be some degree of regularity in this process (Fig. 2), suggesting that the lateral limits on the dimensions of listric normal faults may, in part, control the spacing of accommodation zones. Extension across the rift zone and concomitant lithospheric thinning eventually become large enough to allow complete decoupling of the two now distinct lithospheric plates and the formation of new oceanic crust. At this point, the fault systems beneath opposite sides of the rift also become isolated and develop independent fault geometries.

Recent models for the propagation of continental rifts have attempted to assess the significance of early continental extension in the development of passive continental margins, and how that extension affects plate reconstructions¹⁻⁴. Asymmetries inherent in the rift scenario described above may be reflected in the structural and stratigraphical record of rifts long after oceanic crust first appears. In particular, a geoseismic model, stratigraphical column or thermal maturation curve derived at one point on a passive margin may not accurately apply to rocks a few tens of kilometres along strike, especially with respect to the initial rift sequence. Propagating oceanic rifts^{41,42}, where rift boundaries are almost immediately structurally decoupled, and propagating continental rifts, are undoubtedly quite disparate structural phenomena.

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Three distinct types of dynamic behaviour shown by a single planktonic system

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A wide range of dynamic behaviour has been uncovered by studying disparate predator-prey systems¹, ranging over population stability, cycles and non-cyclic fluctuations. We know of no instance, however, in which qualitatively different dynamics have been found in the same natural predator-prey populations at different times, or in a given predator-prey system found in different but similar communities. Mathematical models of predator-prey systems, by contrast, predict that any particular system may exhibit the whole range of dynamics simply as a result of changes in rate parameters, such as those that might occur at different temperatures. Indeed, May raises the possibility that different *Daphnia*-algal dynamics in the laboratory are caused by differences in the relative values of time lags and rates of increase at different temperatures². Therefore, to produce the different dynamics, the models need not undergo any change in structure, such as might occur in different systems or groups of organisms. Here we show that field populations of a predator, the zooplanktonic herbivore *Daphnia*, and its algal prey exhibit three qualitatively different types of dynamic behaviour. This finding supports the basic premise that different dynamics can result from quantitative differences within a constant structural framework, and hence the hypothesis that variations in dynamics between systems can be explained in the same way as differences within a single system.

Populations of *Daphnia* have been raised in the laboratory under constant additions of food at fixed 2-4-day intervals³⁻⁸. These populations are strictly food-limited, their average density being a linear function of the rate of food supply³. One outcome of these studies is sustained fluctuations in density that are sufficiently regular to qualify as cycles³⁻⁶, with a period of 20-40 days. As the rate of addition of algal food is fixed and, by design, the algae cannot grow during these experiments, the oscillations are a property of *Daphnia* demography; they are single-species rather than predator-prey (or herbivore-plant) oscillations. A second outcome is damped oscillations and a third is the absence of oscillation^{7,8}.

This impressive range of dynamic behaviour in *Daphnia*, despite constant conditions and fixed food supply, led us to survey the published studies of field population dynamics of *Daphnia*, concentrating on those for which data are also available on phytoplankton fluctuations. We restrict ourselves here to discussing the dynamics of 17 studies drawn from a wide diversity of freshwater habitats⁹⁻²³, and include our own data from two experimental populations. We have excluded studies in which competition between congeneric species of *Daphnia*²⁴⁻²⁶ or fish predation²⁷⁻³⁰ appear to be important factors in the dynamics (we will examine the effects of these factors elsewhere). We have not included three examples from very cold lakes in which *Daphnia* has only about one generation a year³¹⁻³³, because the longest of these studies covers only two generations.

An implicit assumption is that the *Daphnia* and the phytoplankton populations in these different habitats have sufficiently similar biology for them to be 'structurally' similar (that is, describable by the same general model), although their parameter values no doubt differ. This is reasonable for *Daphnia* species, which are basically larger or smaller versions of a single template. The phytoplankton in any particular habitat are made up of many species, and the species composition will vary from one habitat to another. Clearly, we cannot be certain that these collections all have qualitatively similar features. The phytoplankton are more homogeneous in important features, however, than their diverse taxonomy might suggest. In most temperate

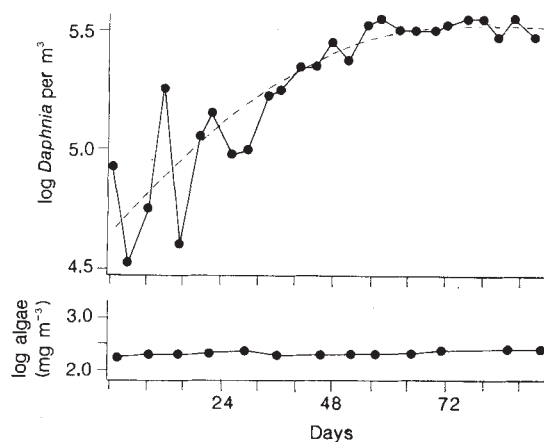


Fig. 1 An example of class 1 dynamics in which $\log_{10}$ *Daphnia pulex* Leydig abundance per m^3 and $\log_{10}$ algal biomass (mg m^{-3}) are stable. The dashed curve is a second-order seasonal trend fitted to $\log_{10}$ *Daphnia* density by least-squares regression analysis ($r^2 = 0.81$, $P < 0.001$). *Daphnia* density deviates little from this seasonal trend; the average residual deviation is $< 15\%$ of the trend value of the population. The experiment was carried out in 1,200-l stock tanks (described elsewhere⁴¹) on the campus of the University of California at Santa Barbara over a 4-month period between June and September (water temperature was $22-26^\circ\text{C}$). These tanks represent a 'natural' habitat in the ranch country of southern California⁴². *Daphnia* populations were sampled every 3-4 days in duplicate (500-ml) samples that were counted in their entirety. Algal biomass was estimated as chlorophyll *a* and converted to cell volume.

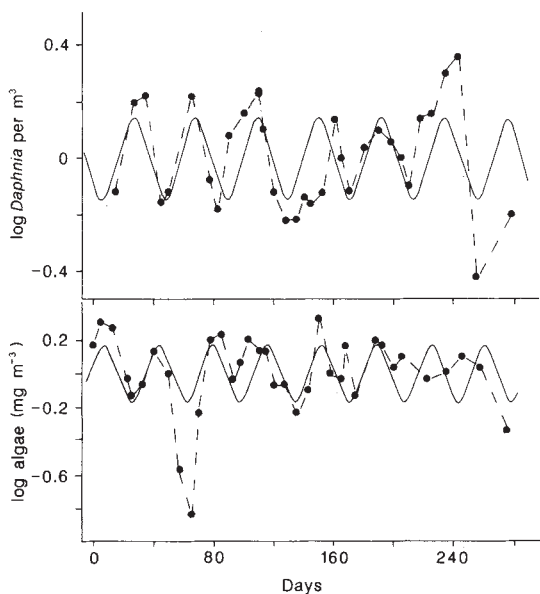


Fig. 2 An example of class 2 dynamics in which *Daphnia* and algae appear to exhibit predator-prey oscillations. These data are from ref. 14 (Table 1). The points joined by the dashed line are the residual deviations from (logarithmic) seasonal trends and the solid line is the sine function fitted to these data (Table 1).

zone lakes, a major proportion of algal biomass is $< 75 \mu\text{m}$, of which *Daphnia* can eat those up to $50 \mu\text{m}$. Indeed, for *Daphnia*, size is a more important feature than taxonomy in distinguishing algae. Edible species vary in growth rate, and perhaps also in nutritive quality, but these variations affect parameters and not structural aspects of the model.

Visual inspection of the data suggested that populations fall into three classes. (1) Stable driven populations. Algal population density is low, with sometimes a peak in autumn in response to an increase in nutrients. *Daphnia* populations usually follow the same pattern, apparently typically being driven by the algae, which in turn are driven by slowly changing environmental factors. *Daphnia* are apparently stable, as their density tracks

Table 1 Results of the analyses of *Daphnia* and algal field populations for the presence of cycles

Ref.	No. of observations (algae/ <i>Daphnia</i>)	Mean	Period (days) 95% c.l.†	P
Class 1				
9,10 (1978)*	49/24	None/none	NA/NA	NA/NA
11	20/15	None/none	NA/NA	NA/NA
12	19/24	213/none	185–243/NA	<0.0001/NA
13	19/29	None/none	NA/NA	NA/NA
Tank experiment	13/27	None/none	NA/NA	NA/NA
Class 2				
14	36/32	36/41	35–38/38–43	<0.05/<0.05
10,15 (1979)*	57/28	39/47	36–41/44–50	<0.05/<0.0001
10,15 (1980)*	23/24	37/45	32–42/41–50	<0.0001/<0.1
16	31/57	47/45	44–50/43–47	<0.05/<0.05
17	25/22	40/28	36–43/26–30	<0.05/<0.05
18	23/50	32/31	29–34/27–34	<0.1/<0.0001
19	22/22	37/36	32–43/32–41	0.0001/NS
20	22/22	32/25	30–33/24–26	<0.05/<0.05
21	26/26	40/36	30–50/29–42	<0.0001/<0.0001
22	24/24	32/36	28–35/29–42	NS/NS
Class 3				
Tank experiment	13/27	None/23	NA/22–25	NA/<0.0001
23	24/24	None/42	NA/38–46	NA/<0.05

A dominant feature of all natural systems is a spring peak and decline in both algae and *Daphnia*, driven by the spring increase in nutrient and light levels, and rising temperature. Our analyses covered the period following this decline. Given the observed range of periods in cycles in *Daphnia* laboratory populations, we did not look for cycles with a period shorter than 20 days; in most cases it was impossible to detect shorter periods given the frequency of sampling in the published studies. Standard time-series analysis was not possible because the data sets were too short, sampling periods were not regular and the two populations were not sampled at the same frequency. Instead, abundances were transformed to logarithms, seasonal trends were removed by fitting linear or second-order regressions³⁹. Populations were considered stable (no cycle present) if the average standard deviation of the logarithm of their densities was <20% of the mean logarithmic density. This percentage is a realistic estimate of sampling variability to be found in such systems, and provides an objective criterion for deciding if a population belongs to class 1. If the average standard deviation of the logarithmic residuals was greater than 20%, the residuals were subjected to Kendall and Stuart's turning point test to detect cycles⁴⁰, and sine functions were fitted using iterative nonlinear regression with parameter estimates from the turning point tests as initial values. In class 1 neither population was cyclical, except that the algae in study 12 showed a long-period seasonal cycle; in class 3 *Daphnia* cycled and algae did not. Each data set contained at least 15 sampling dates and 3 months' data after the spring decline, and almost all had more than 23 observations. Note that in studies from refs 9, 10 and 15, different classes occur in different years in the same system. NA, not applicable; NS, cycle found, not significant.

* Year in which data were collected.

† Confidence limits based on the sequence of periods found in each study.

these slow seasonal changes in food supply and temperature without strong fluctuation. (2) Predator-prey cycles. *Daphnia* and phytoplankton oscillate at close to the same frequency but out of phase, in what seem to be predator-prey cycles. *Daphnia* and algae, more than most herbivore-plant systems, conform to the structure of predator-prey models in that algal individuals are small relative to *Daphnia* and are eaten whole. There is the added advantage that there is no additional basic trophic level, as in true predator-prey systems, which is only implicit in typical predator-prey models. (3) 'Single-species cycles'. *Daphnia* again oscillate, up to six times after the spring decline, but the phytoplankton appear not to oscillate even though they have a very short generation time relative to *Daphnia*. Any fluctuations in phytoplankton that do occur appear unrelated to *Daphnia* fluctuations. This pattern is similar to that observed in some laboratory populations of *Daphnia* provided with a fixed food supply, hence the title for this class.

We confirmed our subjective classification by a series of statistical analyses designed to detect cycles, estimate their period and test their statistical significance. All studies fit unambiguously into one of the three classes (Table 1). Figures 1–3 show one example from each class. Analysis of other populations might of course uncover additional classes of dynamics.

The results provide quantitative confirmation that there are distinguishable classes of population dynamics in the *Daphnia*-algal interaction, and that populations can be either cyclical or non-cyclical. The periods discovered in class 2 and 3 populations (23–47 days at average temperatures of 15–24°C) coincide closely with the range observed in cyclical laboratory populations (temperature range 14–20°C). It would have been possible to detect cycles with periods of almost 100 days in all cases and up to ~200 days in some, so the congruence with laboratory results is probably not fortuitous.

The most common behaviour was predator-prey cycles (class 2) (10 examples). There were five stable driven systems (class 1). After removal of a seasonal trend, these showed either no oscillation or one with a very long period reflecting seasonal change not captured by the simple regression (Table 1). Only two examples were of single-species cycles (class 3), although there are at least three others, for which we do not have the original data, which appear to be class 3^{34,35}.

Two important findings are that different dynamics are sometimes seen in the same lake in different years, and that different classes of dynamics are seen in the same species. The stock tank populations also showed different dynamics in the same species at the same time (Figs 1, 3).

Class 3 dynamics (in which the prey does not oscillate even though it has a very short response time and can oscillate) have not previously been demonstrated to occur in natural populations. Other systems exist in which the predator oscillates and the prey does not, or at least not on a timescale as short as that of the predator, for example, insect pests in forests³⁶ (where the pest is the predator and trees are the prey) and some disease organisms in human populations (where humans are the prey³⁷). Prey oscillations at the same frequency as those of the predator, however, are demographically impossible because of the long generation time of the prey in these situations. (In plankton, by contrast, prey generation time is measured in days, predator generation time in weeks.) The 4- and 10-yr cycles in small mammals could be the predator component of class 3 dynamics. Unfortunately, too little is known about the dynamics of the vegetation to reach a conclusion. In addition, the cycles may be internally driven by individual behaviour, and true predators of the small mammals seem to play a part in the cycles in many cases³⁸.

We cannot explain the apparent absence of oscillations in the

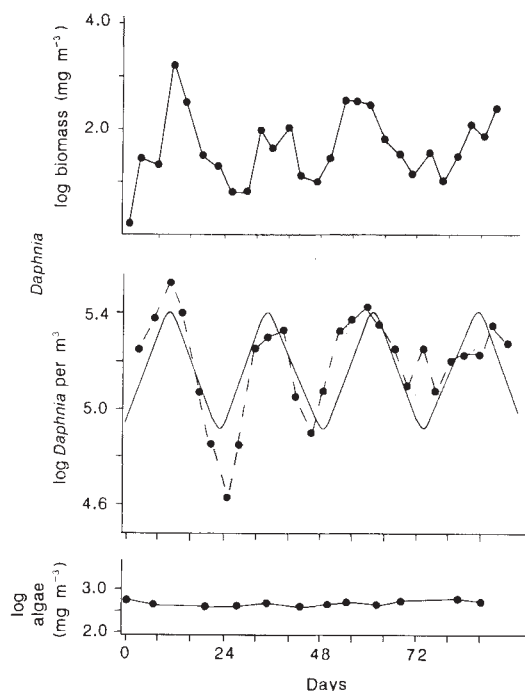


Fig. 3 Class 3 dynamics from tank experiment: log *Daphnia* abundance per m^3 and log *Daphnia* biomass (mg m^{-3}) oscillate, while log algal biomass (mg m^{-3}) is stable. *Daphnia* biomass (dry weight) was estimated using data on abundance and dry weights (derived from length-weight regressions⁴²). These experimental populations were run at the same time as those described in Fig. 1.

algae when they are so clearly marked in the *Daphnia*. We believe the difference between algal and *Daphnia* dynamics is real, but two alternatives, based on the assumption that the difference is an artefact, need to be considered. First, the fluctuations in *Daphnia* density might be caused mainly by changes in age structure, with a roughly constant *Daphnia* biomass being maintained by the roughly constant algal biomass. Figure 3 shows that this is not the case in the one example of this class for which we have detailed information on the sizes of individuals; total biomass also cycles. Second, fluctuations might occur in the edible phytoplankton but would not be seen in the algal data (usually chlorophyll *a* concentration), which reflect the abundance of all algae, edible or not; this implies that the edible and non-edible fractions are negatively correlated in time. This explanation cannot be ruled out for all cases, but it appears not to be valid for Edmondson and Litt's study³⁴ in which the edible fraction was estimated. A third possibility is that the algae do indeed oscillate, but over such a small range that the oscillations are not detected.

Our demonstration that structurally similar systems can exhibit a broad range of dynamic behaviour suggests that it is valid to search for general models which seek to explain the variety of observed dynamic behaviour as resulting from quantitative differences contained within a common structure². In the particular system studied here, our initial hypothesis is that the existence of each broad dynamic pattern can be explained solely by the interaction between *Daphnia* and its food supply. We expect that whether class 1 or class 2 dynamics is observed in a particular lake will be determined by the manner in which factors such as nutrient levels and average temperature influence ecological properties (such as algal rate of increase) of the populations. We are now testing this hypothesis.

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Light-dependent antibody labelling of photoreceptors

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Monoclonal antibodies are tools widely used to analyse the structure of the nervous system. Whereas some labelling patterns are highly reproducible, others appear to vary from one preparation to the next, as we noticed in particular for some antibodies with respect to photoreceptor labelling. To establish whether some of this variability can be linked to functional criteria, we tested for light-dependence. We found two antibodies that label photoreceptor outer segments, only when the retina has been illuminated, and a third antibody that has a selective affinity for dark-adapted outer segments. The two antibodies against light-activated sites are primarily directed against the highly phosphorylated neurofilament subunit at relative molecular mass 200,000 (200K). One of them, RT97, recognizes on immunoblots, in addition to neurofilaments, a light-activated epitope on a protein that resembles the photopigment rhodopsin, presumably a phosphorylation-dependent site. Antibodies like those described here may allow the study of physiological processes such as light and dark adaptation using morphological techniques.

As many physiological events in the nervous system are executed through reversible covalent modifications in molecules, it should be possible to find antibodies selective for particular states of the sites that undergo changes. We found previously that antibodies against the heavy (200K) neurofilament subunit can reveal neuronal reactions to injury; these changes were inducible over a time course of days¹. Some of the same antibodies detect modifications in mitotic cells which appear and vanish within about 1 h (ref. 2). Our studies revealed that two

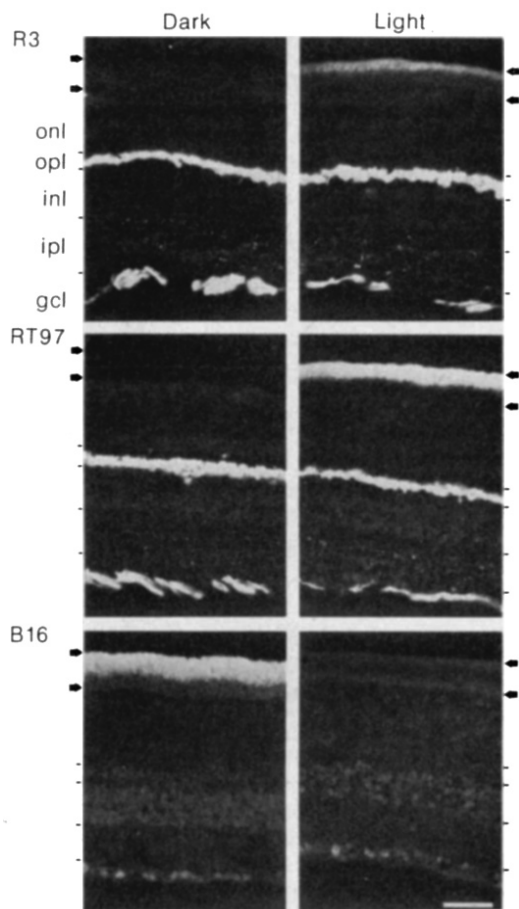


Fig. 1 Transverse sections through dark-adapted (left vertical lane) and light-adapted (right lane) retinas labelled with the antibodies R3 (ref. 4) (top row), RT97 (ref. 3) (middle) and B16 (ref. 6) (bottom). The layer of photoreceptor processes, including inner and outer segments, are indicated by the arrows on the left and right margins; other retinal layers are labelled as: onl, outer nuclear layer; opl, outer plexiform layer; inl, inner nuclear layer; ipl, inner plexiform layer; gcl, ganglion cell layer. Note that the two neurofilament antibodies RT97 and R3 label photoreceptor outer segments only in the light-adapted condition; the labelling of neurofilaments in horizontal cells and optic axons is independent of the illumination history. Antibody B16 has selective affinity for dark-adapted outer segments. Scale bar, 50 μ m.

neurofilament antibodies of the same class, and another unrelated antibody, can be used to detect changes that occur within seconds to minutes in photoreceptor outer segments in response to light and darkness.

C57BL/6J mice were anaesthetized with pentobarbital, their body temperature was maintained at 36.5 °C, and they were dark adapted for 45 min; one eye was taped shut and the other was either diffusely illuminated or was covered with a contact lens and exposed to stationary visual patterns. After ~2–8 min, the mice were quickly perfused under dim red light with 4% paraformaldehyde/0.12% picric acid; the retinas were left as whole mounts or sectioned on a cryostat and processed for indirect immunofluorescence, using fluorescein-isothiocyanate labelled secondary antibodies (Boehringer Mannheim). Of 10 different monoclonal antibodies against 200K neurofilaments that we tested, only two showed light-dependent photoreceptor labelling: the antibodies RT97 (ref. 3) and R3 (ref. 4). The third antibody described here, B16, was generated following the protocol of Matthew and Patterson⁵ in a fusion aimed at obtaining antibodies against photoreceptors⁶: a BALB/c mouse was first immunized with mutant retina lacking photoreceptors (C57BL/6J *le-rd*), then many of the responding B cells were eliminated by intraperitoneal injection of cyclophosphamide, and finally the surviving B cells were challenged *in vitro* with

retina from normal mouse (C57BL/6J), and fused with NS1 myeloma cells⁷.

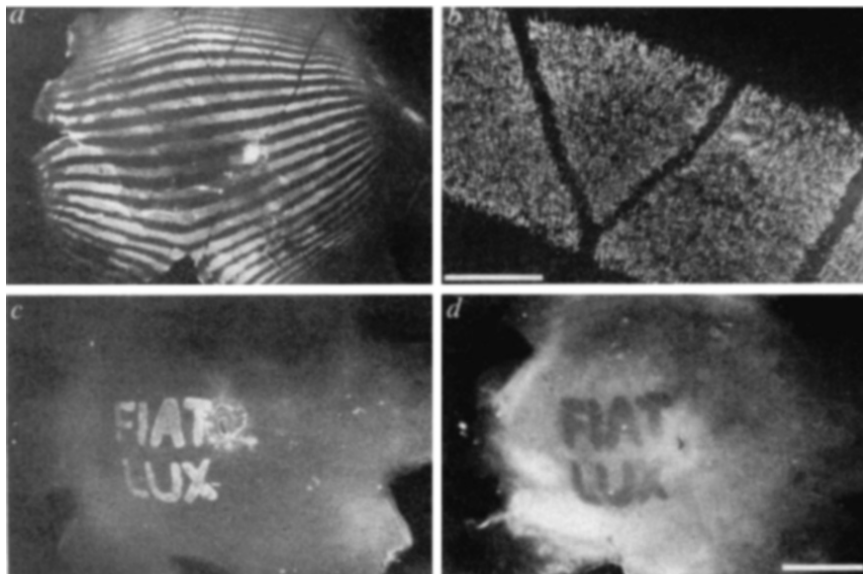
Figure 1 shows sections from dark-adapted (left) and illuminated retinas (right) labelled with these three antibodies. The antibodies RT97 and R3 reveal the characteristic neurofilament distribution which is, as expected, independent of illumination levels: these are components of horizontal, ganglion and amacrine cells. The photoreceptor outer segments, however, are labelled only in the illuminated retinas and negative in the dark-adapted ones. As there are no neurofilaments in outer segments, the light-activated labelling must reflect a chance cross-reactivity. The third antibody, B16, shows the reverse pattern with respect to the outer segments: it labels them only in the dark-adapted state. In addition this antibody labels synaptic components with high affinity and all cell nuclei with low affinity, regardless of the illumination history. All three antibodies appear to label only the outer segments of rods and not of cones, as we determined by double-labelling with cone-specific peanut lectin⁸ (data not shown).

The labelling is spatially precise: if the mouse had been viewing a striped pattern, the retina is labelled in a striped fashion, as shown for RT97 in a whole mount in Fig. 2a. If the mouse was held stationary during visual stimulation the stripes are interrupted by the shadows of the blood vessels in the inner retina (Fig. 2b). To illustrate the complementarity of labelling with the two types of antibodies, we let the mice face writing in bright letters: a retinal whole mount from such a mouse labelled with RT97 shows the writing as bright on dark (Fig. 2c), and in a B16-labelled retina the writing appears dark on a bright background (Fig. 2d).

Although both neurofilament antibodies label outer segments only in the light, they seem to recognize different antigenic sites, as indicated by light-adaptation experiments: anaesthetized mice were exposed to a brightly illuminated screen in the nasal half of their visual field with the temporal half blocked off by a black mask; after 10 min the mask was quickly pulled away to allow for short periods of full-field stimulation. The mice were then rapidly perfused under dim red light, the retinas sectioned dorsally to ventrally and examined for temporal-nasal differences in photoreceptor labelling. Such light-adaptation experiments revealed traces of the RT97 site within as little as 10 s of light exposure; over the following minutes the labelling intensity increased up to a maximum and thereafter remained constant. The R3 site behaved differently: like the RT97 site it turned on rather rapidly in response to light onset and grew to a maximum; however, it then diminished with steady illumination. This is illustrated in Fig. 3 for a mouse whose nasal retina (right) was illuminated for 10 min and 45 s, and the temporal retina (left) for 45 s only: RT97 labels the 45 s exposure less brightly than the 10 min + 45 s exposure, whereas R3 shows the reverse pattern: the retina half illuminated for 45 s is brighter. Hence under the intense illumination conditions used here the R3 site appears to respond preferentially to light transients, whereas the RT97 site seems to measure the amount of illumination.

We tried to determine the relative molecular masses of the illumination-dependent antigens. C57BL/6J mice were light- or dark-adapted for 1 h; their retinas were removed under bright white or dim red light, homogenized and dissolved in cold SDS, separated on SDS/polyacrylamide gels and blotted onto nitrocellulose paper^{9,10}. So far we have found a clear light/dark difference on blots only for RT97, as shown in Fig. 4a, which compares RT97 with the monoclonal antibody RET-P1 that recognizes rhodopsin¹²; the lanes of dark-adapted retinas (D) were loaded with twice the amount of protein as the lanes of the light-adapted preparation (L) to bias against detection of light-activated antigens. RET-P1 labels both in the light- and dark-adapted preparations three broad regions which presumably correspond to rhodopsin and its aggregates; the broad distributions reflect multiple bands that probably differ in phosphorylation states. RT97 reacts practically only with the light-adapted preparation; it recognizes mainly a broad region at

Fig. 2 Whole mounts of retinas from mice exposed to stationary visual patterns for ~8 min, and reacted with RT97 (*a-c*) and B16 (*d*). The mouse of the retina in *a* had been viewing a flat panel with parallel stripes covering most of the visual field through the stimulated eye. The labelling pattern reflects a combination of tangent and spherical distortions. In a higher power view of a different retina (*b*) a stripe, composed of labelled rod outer segments, can be seen disrupted by the shadows of blood vessels from the inner retina, indicating that the mouse did not move during visual stimulation. What at this magnification appears as slight irregularities in outer segment labelling corresponds at higher magnifications to cross-striations within single outer segments: apparently, stacks of disks partially separated because of mild detergent treatment of the whole mounts. Single labelled outer segments are visible here, because they are surrounded by unlabelled ones, which seems to reflect the mode of dark adaptation of the RT97 sites; with increasing time in the dark after a strong light stimulus, increasing numbers of outer segments lose RT97 affinity in an all-or-none fashion. The mice in *c* and *d* were exposed to the same bright writing on a dark background: RT97 recognizes the illuminated letters and B16 the dark background. Under the gentle conditions of antibody incubation used here, RT97 did not penetrate sufficiently into the tissue to label neurofilaments except around the optic disk and at the edges of the whole mounts. Scale bars, 1 mm (*a, c, d*) and 200 μ m (*b*).



33–39K and a weaker one at 67–76K, both of which line up roughly with the two lower regions of RET-P1. In other preparations a third band just above 100K, at the approximate location of rhodopsin trimers, was very weakly labelled in a light-dependent fashion. In preparations aimed at minimizing rhodopsin aggregation (Fig. 4*b*), RT97 recognized a single light-induced band at 34–40K, in addition to light-independent bands that correspond to the 200K neurofilament subunit and its presumed breakdown products¹¹. A plausible explanation is that RT97 crossreacts with light-adapted rhodopsin; if this assumption is correct, the apparently higher affinity of RT97 for rhodopsin monomers than for aggregates, compared with RET-P1, may indicate either that the RT97 site interferes with rhodopsin aggregation or that it is less accessible in aggregates.

We do not yet know what the changes at the three illumination-dependent sites may be, but a cue to the nature of two of them comes from the affinity of the antibodies for 200K neurofilaments. This neurofilament subunit is known to be highly phosphorylated, and most 200K neurofilament antibodies were shown to label neurofilaments in a phosphorylation-dependent manner^{13–15}: they presumably recognize either a short peptide

sequence containing a phosphoserine, or a change brought about in the peptide chain by nearby phosphorylation. A phosphorylation dependent labelling applies also to the two antibodies RT97 and R3 used here: their affinity for neurofilaments can be abolished by alkaline phosphatase treatment (J. Wood, personal communication); we observed the same for isolated outer segment preparations (data not shown). A major covalent modification in photobleached rhodopsin is phosphorylation at a total of nine sites, a process thought to impede its ability to participate in the phosphodiesterase activation cycle and considered a mechanism in light adaptation^{16–19}. For RT97 a crossreactivity with one of the light-induced phosphorylation sites on rhodopsin appears likely. R3 may also cross-react with rhodopsin (although so far we could not detect such a cross-reactivity on blots) or it may recognize one of several other outer segment proteins that are phosphorylated in the light²⁰. The light-adaptation experiments indicate that the R3 site probably differs from the RT97 site and that the two sites may mediate different functions. As activation of the RT97 site increases with illumination time up to a saturated level, its behaviour is consistent with a mechanism involved in the mediation of light adaptation. The R3 site,

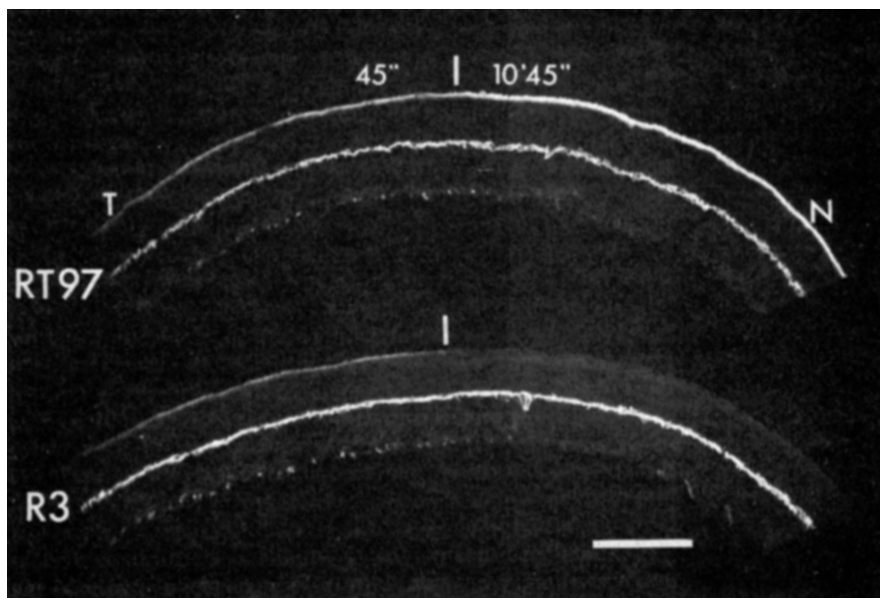


Fig. 3 Light-adaptation experiment. Transverse sections from retina exposed to bright light for 10 min in its nasal half only, and for 10 min and an additional 45 s to full-field stimulation. The border between the nasal (N) and temporal (T) halves is indicated by the white lines above the sections. Note that RT97 labels the 10 min + 45 s exposure more brightly than the 45 s exposure, whereas the R3 labelling is brighter after the shorter stimulation period. Scale bar, 200 μ m.

on the other hand, does not seem suited to mediate light adaptation, as it seems to adapt to light by itself. Alternatively, the apparent light adaptation of the R3 site may be an artefact resulting from steric hindrance of antibody binding through neighbouring additional phosphorylation sites. Finally, we have no clue to the antigen(s) recognized by antibody B16 in photoreceptors. If labelling with B16 were also related to phosphorylation events, it may be directed against one of the components in photoreceptor outer segments that are phosphorylated in the dark in a cyclic GMP-dependent manner, or it may recognize a site that is unmasked by dephosphorylation in the dark^{21,22}.

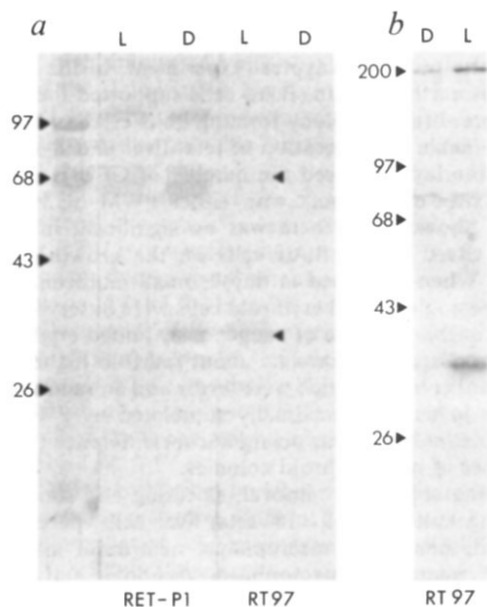


Fig. 4 *a*, Immunoblot of RT97 (ref. 3) on light- and dark-adapted retinas and comparison with RET-P1, a monoclonal antibody recognizing rhodopsin¹². Mice were either light-adapted ($>100\text{ cd m}^{-2}$) or dark-adapted for 1 h, and killed by cervical dislocation. Retinas were quickly removed, homogenized in a buffer containing 1 mM EDTA, EGTA and phenylmethylsulphonyl fluoride, and treated with DNase (1 mg ml⁻¹). Samples were dissolved in cold sample buffer to a final concentration of 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue in 0.625 M Tris HCl, pH 6.8, briefly sonicated (not boiled) and loaded onto a 12% polyacrylamide gel: 100 μg protein from dark-adapted retinas per D-lane, and 50 μg from light-adapted retinas per L-lane. Electrophoresis was performed using the buffer system of Laemmli⁹, and proteins were transferred from the gel onto nitrocellulose paper according to Towbin *et al.*¹⁰. Strips of nitrocellulose paper were either stained for protein with amido black, or blocked with 3% bovine serum albumin and processed for antibody binding using peroxidase labelled secondary antibody (Cappel) and 3,3',4,4'-biphenyl tetramine tetrahydrochloride (Sigma) as chromogen. Relative molecular mass standards were myosin (200K), phosphorylase B (97K), bovine serum albumin (68K), ovalbumin (43K) and α -chymotrypsinogen (26K). Note that RT97 recognizes two broad regions in the light-adapted lane that line up roughly with the presumed monomers and dimers of rhodopsin labelled by RET-P1. Apparent relative molecular masses of the labelled regions, as determined from this and four other gels, were 33–39K (s.d. 3) and 67–76K (s.d. 8) for RT97, in addition to a very faint band just above 100K, and 32–38K (s.d. 3), 67–78K (s.d. 7) and 103–109K (s.d. 1) for RET-P1. *b*, Light- and dark-adapted retinas blotted with RT97, prepared slightly differently to minimize rhodopsin aggregation and to demonstrate neurofilament labelling: one retina per lane was homogenized in 50 μl pH 7.4 Tris buffer containing 1 mM EDTA and 0.1 μl saturated phenylmethylsulphonyl fluoride, and incubated for 10 min with 0.017% sodium deoxycholate; the protein was precipitated by adding 6 μl of 72% trichloroacetic acid and the precipitate was resuspended in SDS sample buffer⁹ by sonication and vortexing, and treated as above. The light-independent bands are the same as seen by Calvert and Anderton¹¹ on blots of rat brain with RT97: the 200K neurofilament subunit and presumed neurofilament breakdown products.

We have shown here that monoclonal antibodies can be used to detect relatively fast physiological changes in photoreceptors. Such antibodies may allow the analysis of reversible covalent modifications at single sites, which at least for the phosphorylation sites on rhodopsin has not been possible with conventional biochemical techniques. As many physiological changes are brought about by phosphorylation events²³, the group of 200K neurofilament antibodies may provide a reservoir of detectors for functional epitopes.

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Stimulation of multipotential, erythroid and other murine haematopoietic progenitor cells by adherent cell lines in the absence of detectable multi-CSF (IL-3)

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It is well established that murine multipotential and committed erythroid progenitor cells require the presence of a glycoprotein, termed multi-CSF (multi-colony-stimulating factor, IL-3)^{1–7} for clonal proliferation and differentiation *in vitro*. The initial proliferation of these cells can also be stimulated by two other glycoproteins, granulocyte-macrophage CSF (GM-CSF) and granulocyte CSF (G-CSF), although continued proliferation and differentiation requires the subsequent presence of multi-CSF^{8,9}. Here we report the stimulation of multipotential, erythroid and other haematopoietic progenitor cells by a number of adherent cell lines including a cloned bone marrow cell line (B.Ad). The positive cell lines, as feeder layers, exhibit colony-stimulating, erythropoietin-like and burst-promoting (BPA) activities. Optimal erythropoietic stimulation by the B.Ad line requires close cell-cell contact. The cell lines also support the *in vitro* clonal growth of multipotential colony-forming cells and progenitors of six other haematopoietic lineages. The biological activities observed seem not to be mediated by known multipotential or erythroid colony-stimulating factors (multi-CSF⁷, IL-3⁵, MCGF¹⁰, HCGF³, PSF⁶, BPA²).



Fig. 1 Phase contrast photograph showing confluent cultures of B.Ad cells. Note clusters of polygonal cells heavily laden with lipid droplets. $\times 135$. The cell line was isolated from a non-adherent cell population taken from a 1-week-old liquid culture of BALB/c bone marrow cells. It was observed during weekly serial passages of the non-adherent expanding population of mast cells in growth medium (DMEM containing 20% fetal calf serum, supplemented with medium conditioned by WEHI-3B myelomonocytic leukaemic cells (WEHI-3B CM)), that there were always adherent cells growing in the cultures. On reaching confluency, cells heavily laden with lipid droplets appeared. Nine months after initiation of the cultures, non-adherent cells were removed and adherent cells collected by trypsinization and cultured alone. This cell line has been designated B.Ad (BALB/c adherent) and has now been maintained as adherent cultures for >1 yr.

B.Ad cells were derived from BALB/c bone marrow adherent cell cultures (see Fig. 1 legend). On reaching confluence, cultures of B.Ad cells displayed clusters of lipid-containing cells intermixed with fibroblastoid cells (Fig. 1). The lipid-containing cells were oil red O positive, and most cells showed weak nonspecific esterase activity, but no metachromasia, myeloperoxidase or acetylcholinesterase activity. Approximately 95% of the cells phagocytosed latex particles and had the following surface phenotype: $H-2^+$ (40–60%), Ia^- , Ig^- , $Lyl-2^-$. The mean population doubling time was 76 h and the cells had a modal chromosome number of 75. Tests for tumorigenicity are in progress,

but no tumours are evident 2 months after subcutaneous inoculation of 10^6 B.Ad cells into syngeneic newborn mice.

Table 1 shows colony formation by bone marrow cells separated from the adherent layer of B.Ad cells by an intermediate layer of agar medium. Most colonies were composed of neutrophils and/or macrophages but megakaryocytes and mixed cell colonies were also observed (average of 4 per culture), although less colonies were stimulated by the B.Ad cells than in control cultures stimulated with PWM-SCM (pokeweed mitogen-stimulated murine spleen cell-conditioned medium).

The erythropoietic stimulating activity of B.Ad cells was investigated using methylcellulose cultures which allow target fetal liver cells to seed directly on top of the feeder layers. Table 2 shows the result of a typical experiment. In the absence of exogenous erythropoietin, B.Ad cells supported the growth of late erythroid (day 2 colony forming units-erythroid (CFU-E)) colonies (Table 2). Separation of fetal liver and B.Ad cells by an agar interlayer reduced the number of CFU-E colonies by 75%. Mixing experiments with either PWM-SCM or erythropoietin showed that there was no significant inhibitory or additive effect by the B.Ad cells on the growth of CFU-E colonies. When examined at day 7, small multicentric foci of poorly haemoglobinized erythroid cells were observed. In sharp contrast, in the presence of exogenously added erythropoietin, the B.Ad cells could stimulate about fourfold higher numbers of erythroid colonies which were larger and more haemoglobinized than in cultures maximally stimulated by PWM-SCM + erythropoietin. There was no significant difference observed in the number of non-erythroid colonies.

Following sequential removal, smearing and staining of all colonies in cultures of 2×10^3 fetal liver cells, pure erythroid, neutrophil, neutrophil-macrophage, neutrophil-macrophage-mast cell, neutrophil-macrophage-eosinophil and erythroid-neutrophil-macrophage-eosinophil-mast cell-megakaryocyte-blast cell colonies were observed.

The fetal liver origin of the macroscopic erythroid colonies was confirmed by the presence of a single adult haemoglobin band ($\alpha_2\beta_2$ major) characteristic of C57BL/6 mice, when C57BL/6/f/J WEHI fetal liver cells were seeded onto the B.Ad cell line.

Separation of the target cells from the adherent cells by an agar interlayer reduced the number of macroscopic erythroid colonies by $>90\%$ but did not significantly affect the number of non-erythroid colonies. In cultures containing both B.Ad cells and PWM-SCM the same high numbers of erythroid colonies developed as with B.Ad cells alone, suggesting that no inhibitors

Table 1 Morphological characterization of murine bone marrow colonies stimulated by feeder layers of B.Ad cells

Stimulus	Total colonies	% Colonies§					
		Neutrophil	Neutrophil-macrophage	Macrophage	Megakaryocyte	Erythroid (pure and mixed)	Mixed-cell
PWM-SCM*	163 $\pm$ 15‡	8.8	43.9	29.0	11.2	5.9	1.0
B.Ad†	106 $\pm$ 7	21.6	62.6	11.9	3.1	0.0	0.9

50,000 CBA/CaH bone marrow cells were seeded into 35-mm Petri dishes containing 1 ml 0.3% agar in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum (FCS). Cultures were kept in a fully humidified 37 °C incubator containing 10% CO₂ in air. Cultures were scored after 7 days of incubation and were then fixed with 2.5% phosphate-buffered glutaraldehyde at room temperature overnight. Whole culture mounts were prepared and stained sequentially with cholinesterase stain, Luxol-fast-blue stain and counterstained with Meyer's haematoxylin. Colonies were typed according to criteria described previously¹⁶.

* PWM-SCM was prepared from BALB/c spleen cells as described previously¹⁷; 0.1 ml of pre-determined dilution of PWM-SCM (giving maximal stimulation) was used.

† Monolayers of B.Ad cells were prepared in 35-mm Petri dishes by splitting confluent stock cultures 1:100 and seeding cells in 1 ml medium. On reaching confluency (10–14 days), culture medium was removed and 0.5 ml 0.3% agar medium was laid on top of the cell monolayer. After gelling, 1 ml of agar medium containing bone marrow cells was overlaid and allowed to gel before incubation.

‡ Triplicate counts (means $\pm$ s.d.).

§ Results were the average of triplicate plates.

|| Mixed-cell colonies containing neutrophils, macrophages, either eosinophils or megakaryocytes or both with the apparent absence of recognizable erythroid cells.

Table 2 Increased erythroid colony formation by close contact with feeder layers of B.Ad cells

Stimulus	No. of colonies per culture					
	-Epo			+Epo		
	Day 2 CFU-E	Erythroid	Day 7 Non-erythroid	Day 2 CFU-E	Erythroid	Day 7 Non-erythroid§
PWM-SCM	324	11	63	798	17	61
MLCM*	0	0	57	828	5	32
Medium	0	0	0	870	0	0
B.Ad uncloned line	228	0	73	840	80	43
B.Ad uncloned line (A)†	54	0	59	888	6	66
B.Ad uncloned line + SCM	330	6	78	864	87	63
B.Ad uncloned line (A) + SCM	360	2	78	942	ND	ND
B.Ad‡						
Clone 2	144	0	62	828	110	37
Clone 2 (A)	72	0	54	936	8	50
Clone 3	192	0	61	756	153	35
Clone 3 (A)	36	0	56	828	5	60
Clone 4	156	19	74	648	75	37
Clone 4 (A)	12	0	65	876	6	52
Clone 6	48	0	67	456	94	44
Clone 6 (A)	24	0	53	840	4	70
Clone 7	108	0	60	528	105	44
Clone 7 (A)	72	0	55	948	7	70

Adherent layers of B.Ad cells were prepared as described in Table 1; 10,000 12-day CBA/CaH fetal liver cells were cultured in 35-mm Petri dishes in 1 ml volume of 0.78% methylcellulose (Methocel MC, premium, 4,000 cP, Dow) in Iscove's modified Dulbecco's medium containing 20% fetal calf serum and 7.5×10^{-5} M 2-mercaptoethanol. Cultures were kept in a fully humidified incubator at 37 °C with 5% CO₂ in air. CFU-E colonies were scored on day 2 and both erythroid and non-erythroid colonies were scored on day 7. Erythropoietin (Epo) was partially purified from human urine and has been shown to be free of CSF activity. 0.1 ml of predetermined dilution of erythropoietin (giving optimal stimulation of CBA fetal liver CFU-E colonies) was used. ND, not determined, or not scored.

* MLCM, mouse lung conditioned medium, was prepared as described previously¹⁸, 0.1 ml of pre-determined dilution of MLCM (giving maximal stimulation of colony growth by bone marrow cells) was used.

† A 0.5 ml, 0.3% agar-medium, interlayer was laid on top of the B.Ad feeder layers before methylcellulose medium containing target cells was plated. The presence of an interlayer neither inhibited nor enhanced colony formation in control cultures stimulated with PWM-SCM.

‡ B.Ad cells were cloned either by limit dilution method in 96-well tissue culture plates (at a density of 0.4 cells per well in 0.2 ml growth medium) or by single-cell manipulation of B.Ad cells in the log phase of growth. In the latter method, diluted cell suspensions of B.Ad cells were prepared and seeded into 15-cm Petri dishes containing pieces of coverslips. After 1 h of incubation, coverslips with single cells or doublets were located under an inverted microscope and were individually removed and transferred to 12-well tissue culture plates or 35-mm Petri dishes for clonal expansion. A total of 12 subclones was successfully derived.

§ In the absence of erythropoietin, degenerating erythroid colonies are included in the non-erythroid category. In the presence of erythropoietin degenerating erythroid colonies were rarely observed. The apparent increase in non-erythroid colony numbers after inclusion of agar interlayer reflects difficulty in distinguishing non-erythroid colonies intermixed with B.Ad cells in cultures without agar interlayer.

were present in PWM-SCM preventing colony formation by the erythroid progenitors.

Several clones of the B.Ad cell line were isolated and all exhibited biological activities similar to those associated with the parental uncloned line (Table 2).

To determine whether the observed biological activities could be attributed to multi-CSF⁷ (IL-3), we investigated whether feeder layers of B.Ad cells could support colony formation by the multi-CSF-dependent cell line, 32D cl.3 (ref. 11). It was observed that the B.Ad cells could neither support the clonal growth in semi-solid cultures (Table 3) nor the survival of 32D cells in liquid co-cultures. Furthermore, prolonged incubation of haematopoietic cells (either bone marrow or spleen) with B.Ad cells for up to 4 weeks did not result in the generation of suspension cultures of mast cells.

We next investigated whether medium conditioned by B.Ad cells or B.Ad cells and fetal liver cells contained the biological activities described previously. Even after 18-fold concentration, we could not detect erythroid, megakaryocyte, mixed cell or 32D cl.3 colony stimulating activity in the conditioned medium, although macrophage and to a lesser degree, neutrophil colonies were stimulated in agar cultures of bone marrow cells. However, when conditioned medium was added to liquid cultures for 7 days containing 3-day post-5-fluorouracil-treated bone marrow

Table 3 Failure of B.Ad cells to support the clonal growth of 32D cl.3 cells

Stimulus	No. of colonies per culture
WEHI-3B CM*	57 ± 6
WEHI-3B CM (A)†	61 ± 7
Normal saline	0 ± 0
Normal saline (A)	0 ± 0
B.Ad uncloned line	0 ± 0
B.Ad uncloned line (A)	0 ± 0
B.Ad uncloned line + WEHI-3B CM	61 ± 6
B.Ad uncloned line (A) + WEHI-3B CM	70 ± 6

32D cl.3 cells were maintained by passage in DME containing 10% FCS with 10% WEHI-3B CM as a source of growth stimulus. For assay, cells were washed three times and cultured at a density of 100 cells ml⁻¹ in DMEM containing 0.78% methylcellulose and 20% FCS. Cultures were incubated at 37 °C in an atmosphere containing 10% CO₂ in air. Colonies were scored on day 7.

* WEHI-3B CM, medium conditioned by WEHI-3B myelomonocytic leukaemic D⁺ cells.

† 0.5 ml of 0.3% agar medium was laid either in blank Petri dishes or on top of the feeder layers of B.Ad cells before 32D cells were plated.

Table 4 Comparison of B.Ad and other adherent cell lines for ability to stimulate erythroid colony formation

Stimulus	No. of colonies per culture			
	-Epo		+Epo	
	Day 2 CFU-E	Day 7 erythroid	Day 2 CFU-E	Day 7 erythroid
PWM-SCM	180	17	432	29
Medium	0	0	552	0
B.Ad clone 2	36	2	408	63
BALB/c 3T3	12	6	348	45
Swiss 3T3	24	0	396	42
RAT-2	8	0	172	25
WEHI-265	1	0	52	0
LTA-5	0	0	408	0

Adherent cells seeded at 1×10^5 cells in 35 mm Petri dishes, 24 h later 10,000 12-day CBA/CaH fetal liver cells in methylcellulose medium cultured above adherent cells as described in Table 2.

cells, generation of multipotential and progenitor cells occurred. The latter were assayed in agar cultures containing PWM-SCM and were present at three- to fourfold higher numbers in liquid cultures containing B.Ad conditioned medium than control liquid cultures containing PWM-SCM. No cells were detected in liquid cultures containing mouse lung conditioned medium as a source of GM-CSF.

The erythroid potentiating activity of the B.Ad cells was compared with five other adherent cell lines (Table 4). Three of these lines supported day 7 erythroid colony formation in the presence of erythropoietin and two were not active in this assay. A sixth cell line (NIH 3T3) did not support day-7 erythroid colony formation, although its growth was variable in the presence of the fetal calf serum required for erythroid colony growth. None of these cell lines produced detectable multi-CSF when assayed with 32D cells, but one (Swiss 3T3) stimulated a GM-CSF⁻, multi-CSF-dependent cell line, suggesting the presence of GM-CSF.

The present experiments suggest that the erythroid colony stimulating activity associated with the B.Ad cells was not mediated by multi-CSF and unlikely to result from GM-CSF. Evidence against the former comes from lack of stimulation of 32D cl.3 cells, and the fourfold increase in erythroid colonies (compared with PWM-SCM-stimulated controls) and presence of megakaryocyte colonies would not be expected with GM-CSF¹². The results suggest the presence of a new as yet unidentified factor having the biological effects observed. The ability of B.Ad conditioned media to stimulate haematopoietic cell generation in liquid cultures of 5-fluorouracil-treated bone marrow cells may provide a convenient method to assay this factor. A similar conclusion has been made recently using conditioned media from a number of uncloned bone marrow cell lines, or a human bladder carcinoma cell line^{14,15}.

Another possible explanation for the unexpected range of colony stimulating activities expressed by the B.Ad cells could be via stimulation of accessory cells present in the fetal liver target cell population. However, identical results have been obtained using uncloned and cloned B.Ad cells with purified populations¹³ of fetal liver progenitor cells (data not shown) and no appropriate activity was detected in medium conditioned by mixtures of B.Ad and fetal liver cells.

The present experiments also suggest that there is an additional population of erythroid progenitor cells present in fetal liver that cannot be stimulated by multi-CSF and that the stimulation is more effective if target cells and adherent cells are in close contact.

The ability of several other cell lines to stimulate day 7 erythroid colony formation in the apparent absence of multi-CSF requires further investigation. The fact that several cell lines were inactive suggests that the erythroid effect is not caused by improvement of culture conditions by some general metabolic

effect. However, the positive cell lines should provide a convenient source of conditioned media to aid identification of the proposed new factor(s) producing the reported biological effects.

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Nonrandom loss of chromosome 15 in Syrian hamster tumours induced by v-Ha-ras plus v-myc oncogenes

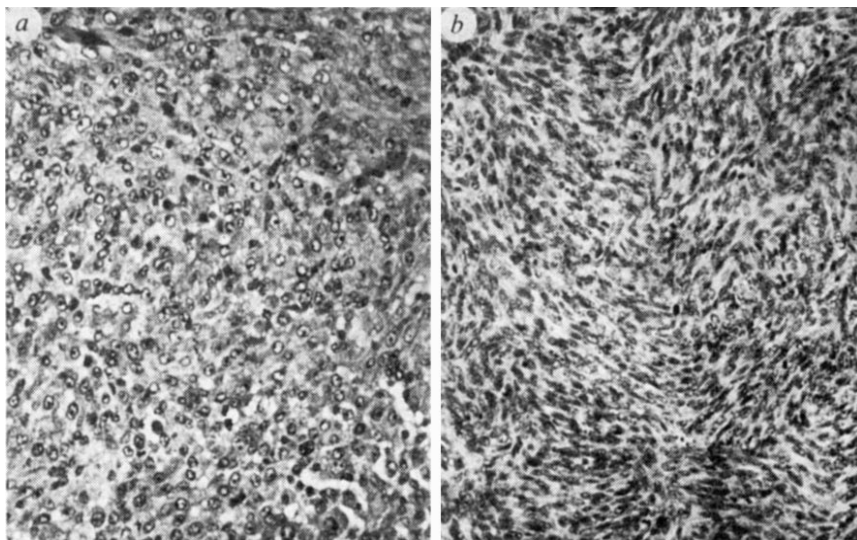
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Nonrandom chromosome rearrangements, observed in a variety of human and animal tumours^{1,2}, are associated in some cases with enhanced expression or deregulation of cellular oncogenes^{3,4}. Recently, it was shown that normal, diploid rodent cells are neoplastically transformed following transfection with two cooperating oncogenes, for example *myc* plus *ras*⁵⁻⁷. However, the number of steps necessary to convert a normal cell into a malignant cell is unknown. If activation of two oncogenes is sufficient for tumorigenicity, tumours derived from diploid cells transformed by the transfected oncogenes may remain diploid or have only random chromosome alterations. We have performed cytogenetic analyses of tumours formed after transfection of Syrian hamster embryo cells with either v-Ha-ras plus v-myc DNAs or polyoma DNA alone. Whereas polyoma-induced, tumour-derived cells were diploid, tumours induced by v-Ha-ras plus v-myc oncogenes were monoclinal and had a nonrandom chromosome change, monosomy of chromosome 15. Thus, an additional change, loss of chromosome 15, is required for or is advantageous for tumorigenicity induced by v-Ha-ras plus v-myc oncogenes. These results suggest that the neoplastic progression of normal, diploid cells requires more than two steps under certain conditions.

Early passage Syrian hamster embryo cells^{7,8} were transfected by the calcium phosphate precipitate technique^{7,9}. Cells treated with v-Ha-ras or v-myc DNA alone (see Fig. 1 legend) were repeatedly non-tumorigenic in nude mice, whereas cells co-transfected with v-Ha-ras and v-myc DNAs and injected 3 days later into nude mice formed tumours at 45-100% of the sites

Fig. 1 Histology of v-Ha-ras plus v-myc-induced tumour (2) diagnosed as pleomorphic sarcoma or fibrosarcoma with marked pleomorphism (a) and polyoma-induced tumour (4) diagnosed as a well-differentiated fibrosarcoma (b). Haematoxylin/eosin staining. ($\times 100$).



Methods. Tertiary cultures were established from 13-day-old Syrian hamster embryos as described previously⁸. Pools of primary cultures derived from littermates were cryopreserved in liquid nitrogen and secondary cultures were initiated from the frozen stocks. All experiments were performed with tertiary cultures. Plasmids [H-1 (ref. 26), pSVmyc⁵ (ref. 5) and PY1 (ref. 10)] containing genomic clones of Harvey murine sarcoma virus (v-Ha-ras), MC-29 viral DNA (v-myc) or polyoma virus DNA, respectively, were added to recipient cells by the calcium phosphate precipitate technique^{7,9}. Cells (3×10^5 per dish) were plated into 10 dishes overnight and then transfected as described with 1 μ g plasmid DNA per dish and 5 μ g of carrier calf thymus DNA. Three days after treatment, cells from each group were pooled and injected into nude mice or syngeneic newborn (3-day-old) Syrian hamsters at a dose of $4-6 \times 10^6$ cells per site and monitored for the appearance of subcutaneous tumours. No tumour formed in animals inoculated with cells treated with calf thymus DNA alone⁷. In contrast, cells treated with v-Ha-ras/v-myc or polyoma DNA formed tumours generally after short latency period (3-7 weeks). Tumours which formed were removed and fixed and histological sections prepared. Tumours also were minced into small (1 mm³) fragments, digested with 0.1% trypsin for 15 min, and re-established in culture.

injected, generally with a latency of 3-5 weeks⁷. These tumours, which were diagnosed as pleomorphic sarcomas or fibrosarcomas (Fig. 1a), grew progressively, reaching more than 3 cm in diameter in 2-3 weeks after detection, and were transplantable in nude mice. Cytoplasmic RNA dot-blot analysis indicated that the cells derived from the tumours expressed both v-Ha-ras and v-myc oncogenes (Fig. 2). Syrian hamster embryo cells treated with the plasmid PY1 containing the three polyoma genes¹⁰ (large-T, middle-T and small-T) were also tumorigenic, forming tumours at 21 of 22 sites injected⁷. The tumours from polyoma-treated cells had essentially the same latency period (4-7 weeks) in nude mice as the v-Ha-ras plus v-myc-induced tumours, but grew at a slightly slower rate, requiring 4-6 weeks to reach 2-3 cm in diameter; these tumours were diagnosed as well-differentiated fibrosarcomas (Fig. 1b). Tumour-derived cells expressed polyoma sequences (Fig. 2) and were readily transplantable into nude mice. Both polyoma-treated and v-Ha-ras plus v-myc-treated Syrian hamster embryo cells also formed tumours when injected into syngeneic newborn hamsters.

Q-banded karyotypic analyses^{11,12} of cells derived from six v-Ha-ras plus v-myc-induced tumours and five polyoma-induced tumours were performed at the first passage (Table 1). The modal karyotypes of all five polyoma-induced tumours (four isolated from nude mice and one from a newborn hamster) were normal diploid (Table 1, Fig. 3a), although 27-40% of the metaphase spreads had abnormal karyotypes, consisting of structural or numerical changes in four out of five of the tumours. However, no consistent change was observed in metaphases from the same tumour and no common changes were observed among tumours. Defendi and Lehman have reported that tumour-derived Syrian hamster cells transformed by polyoma virus apparently remain diploid¹³, but that random chromosome changes in these cells occur with time. Others have reported nonrandom chromosome changes in polyoma virus-transformed Syrian hamster cells several months after infection¹⁴.

Although all the karyotypes of the parental Syrian hamster embryo cells examined were normal, diploid (Table 1), a mixture of XX (61%) and XY (39%) cells were present as the cultures were established from pools of cells obtained from littermates of both sexes. Therefore, sex chromosomes and marker chromosomes were used as markers to determine whether the tumours were monoclonal or polyclonal in nature. Three of five of the polyoma induced tumours had cells with both XX and XY karyotypes (Table 1), indicating that the tumours were polyclonal in origin.

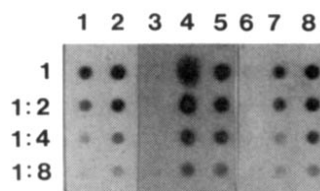


Fig. 2 RNA dot-blot analysis of polyoma and v-Ha-ras plus v-myc tumour-derived cell lines. Cytoplasmic extracts prepared from polyoma-1 (lane 1) and polyoma-2 (lane 2) tumour-derived cells were dotted onto a nitrocellulose filter and hybridized with ³²P-labelled polyoma DNA insert from clone PY1 (ref. 10). Cytoplasmic extracts prepared from Syrian hamster embryo cells (lanes 3, 6), ras/myc-1 (lanes 4, 7) and ras/myc-2 tumour cells (lanes 5, 8) were dotted onto nitrocellulose filters and hybridized with either the ³²P-labelled v-Ha-ras insert from clone BS9 (ref. 27) (lanes 3-5) or the ³²P-labelled v-myc insert from clone pBRmyc²⁸ (lanes 6-8).

Methods. Extracts from 2×10^6 tumour-derived cells or normal cells prepared by the method of White and Bancroft²⁹, and four aliquots representing $0.25-2.0 \times 10^5$ cell equivalents were applied to a nitrocellulose sheet using a Hybri-Dot Manifold (BRL). The filters were heated at 80 °C *in vacuo* for 2 h and hybridized to DNA which had been ³²P-labelled by nick-translation ($1-2 \times 10^8$ c.p.m. μ g⁻¹). Hybridizations were performed in 1 $\times$ Denhardt's solution (0.2% each of bovine serum albumin, Ficoll and polyvinylpyrrolidone), 5 $\times$ SSC (1 $\times$ SSC is 0.15 M sodium chloride, 0.015 M sodium citrate), 50% formamide, 50 mM potassium phosphate (pH 6.5), 0.25% SDS, 200 μ g ml⁻¹ salmon sperm DNA, and 10^6 c.p.m. ml⁻¹ for 48 h at 42 °C. Filters were washed in 0.1 $\times$ SSC, 0.1% SDS at 50 °C and autoradiographed.

In contrast to the polyoma-induced tumour cells, the cells from the v-Ha-ras plus v-myc-induced tumours were karyotypically abnormal (Table 1). All of the cells from the ras/myc-1 tumour were of XX origin and had two common chromosomal abnormalities in all of the metaphase spreads: monosomy 15 and a translocation (X^t) between an X chromosome (at the breakpoint Xpa7) and an unidentified chromosome. However, detailed banding analysis revealed that this unidentified chromosome segment is quite distinct from chromosome 15. Half of the cells had two X^t chromosomes (the modal karyotype of the tumour cells was 44, XX^tX^t, -15) (Fig. 3b). These results suggest that the ras/myc-1 tumour is monoclonal and X^t and -15 are early karyotypic changes. The vast majority of ras/myc-2 tumour

Table 1 Cytogenetic findings in Syrian hamster tumours induced by v-Ha-ras plus v-myc or polyoma oncogenes

Normal or tumour cells	Oncogene transfected	Latency (weeks)	Modal chromosome no.	Modal karyotype	Common chromosome changes	Sex chromosome distribution (XX:XO:XY)*	Remarks
SHE (Normal)	None	>25	44	44,XX	-	61:0:39	Parental cells. No cells with abnormal karyotype observed
PY1	Polyoma	6	44	44,XX	-	49:0:1	40% of the cells have abnormal karyotype
PY2		4	44	44,XX	-	29:0:21	No cells with an abnormal karyotype
PY3		7	44	44,XY	-	0:0:50	27% of the cells have abnormal karyotype
PY4		5	44	44,XY	-	8:0:42	30% of the cells have abnormal karyotype
PY5†		4	44	44,XX	-	0:0:50	36% of the cells have abnormal karyotype
<i>ras/myc-1</i>	v-Ha-ras + v-myc	5	44	44,XX ^t X ^t , -15	-15,X ^t	50:0:0	No cells with a normal karyotype
<i>ras/myc-2</i>		5	42	42,X,-X,-15	-15	11:37:2	Cells with XY chromosome were normal§
<i>ras/myc-3‡</i>		21	42	None of the cells had the same karyotype	-10,-11,-15	0:0:50	No cells with normal karyotype
<i>ras/myc-4†</i>		4	45	46,Y,+1,+14,-15,+19,t(Xq;9p),6q+	-15,t(Xq;9p)	0:0:50	Two cells were 44,XY; tumour formed in newborn male hamster§
<i>ras/myc-5‡</i>		7	43	43,X,+3,-15	-X or -Y,+3,-15	0:50:0	90% of the cells had double minute chromosomes. No cells with normal karyotype
<i>ras/myc-6‡</i>		4	43	43,XY,-15	-15	0:0:50	No cells with a normal karyotype

Tumours were induced following transfection with the indicated plasmids, as described in Fig. 1 legend. Tumour-derived cell lines were established in IBR-modified Dulbecco's Eagle's reinforced medium (Gibco) supplemented with 3.7 g l⁻¹ sodium bicarbonate, 20% (v/v) HyClone fetal bovine serum (Sterile Inc., Logan, Utah), 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin (Gibco). Chromosome slides were prepared by the method described previously³⁰ from the tumour-derived cell cultures at first passage. For chromosome counts, the Giemsa-stained slides were used. For banding analyses, 50 Q-banded metaphase spreads were photographed and analysed. Common chromosome changes are abnormalities observed in all the cells with an abnormal karyotype.

* In *ras/myc-1*, half of the cells (25/50) contained XX^t sex chromosomes; the X^t was duplicated in the remaining 25 cells, that is, XX^tX^t. Thus, all 50 cells were considered to be of XX origin. In *ras/myc-2*, the 11 cells with an indicated XX constitution included 10 with XX and 1 with XXX. In *ras/myc-4*, all of the 50 cells contained a Y chromosome and 48 of 50 cells had an abnormal X chromosome, that is, del(Xq); the deleted segment was probably translocated to the short arm of chromosome 9. Thus, all the cells were considered to be of XY origin.

† Tumours formed in syngeneic newborn Syrian hamsters.

‡ Karyotypic analyses of cells obtained directly from the tumours and/or from tumour-derived cell cultures at first passage. Both methods showed similar karyotypic results; the data from the direct preparation are shown.

§ A small percentage (4%) of the *ras/myc-2* and *ras/myc-4* tumours showed a normal diploid karyotype (44,XY); these cells may have been of host origin (in the case of *ras/myc-4*) or may represent a rare subpopulation of normal diploid tumour cells, or non-tumour cells that exist in the tumour.

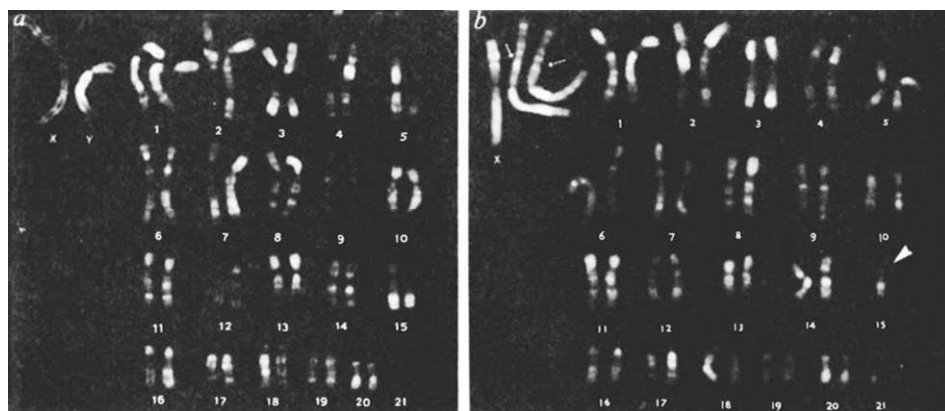
cells had also lost a chromosome 15; out of 50 cells examined, monosomy of chromosome 15 was the sole karyotypic change in 11 cells and 37 cells were monosomic for both the X chromosome and chromosome 15. Two of the cells had a normal karyotype (44,XY) (Table 1). Because 96% of the cells were monosomic for chromosome 15 and in some cases this was the sole karyotypic change, loss of chromosome 15 was probably the first karyotypic change in the transformed cells, with loss of the X chromosome an additional change, which occurred later.

The *ras/myc-3* tumour had a longer latency period than the other tumours and the karyotypes of the cells were more diverse than those of the other tumour-derived lines. No modal karyotype was evident; however, all cells displayed karyotypic alterations and losses of chromosomes 10, 11 and 15 were common changes in all of the metaphase spreads examined (Table 1). The *ras/myc-3* tumour was also examined after a culture period

of only 2 hours to obtain mitotic cells directly from the tumour; the karyotypes of these cells had the same common abnormalities as the cells at first passage. Cells from the *ras/myc-4* tumour, which formed in a male, newborn hamster, were monosomic for chromosome 15 and had a translocation between the X chromosome and chromosome 9, that is, t(X;9) (qa5-7;pa4) (Table 1). Two of the 50 cells examined were normal 44,XY but these may have originated from the male host. All the cells from the *ras/myc-5* tumour showed the absence of an X or Y chromosome and monosomy 15, together with an additional chromosome 3. No karyotypically normal cells were observed. This tumour was also examined when it was still relatively small (~5 mm in diameter) and all the cells analysed directly from the tumour showed monosomy of chromosome 15. All *ras/myc-6* tumour cells had lost a chromosome 15 and no cells with a normal karyotype were observed.

The results presented here indicate that, in contrast to the

Fig. 3 *a*, Q-banded normal, diploid karyotype of a polyoma-induced tumour (2) at first passage. *b*, Q-banded karyotype of a cell with 44,XX^tX^t-15 karyotype in *ras*/*myc*-1 tumour at first passage. The two arrows indicate two X^t chromosomes, and the arrowhead indicates a missing chromosome 15. The translocation (X^t) was between the X chromosome (at the breakpoint Xpa7) and an unidentified chromosome. In this cell, two X^t chromosomes are seen. Chromosome slides were prepared by the method described previously³⁰. The cells were banded with the quinacrine fluorescence dye for karyotypic analysis¹¹. The nomenclature of Syrian hamster chromosomes proposed by Li *et al.*¹² was used.



findings with the polyoma-induced tumours, the v-Ha-*ras* plus v-*myc*-induced tumours were karyotypically abnormal and the cells from all six tumours had a nonrandom chromosome change, that is, monosomy of chromosome 15. Furthermore, the presence of a single sex chromosome and marker chromosomes in the v-Ha-*ras* plus v-*myc*-induced tumours indicates that most of the cells in the tumours are monoclonal in nature. The monoclonality of the tumours may result from a low efficiency of transfection. However, the cells used in the experiments efficiently express pSV2neo DNA following transfection⁷ and the majority of the polyoma-induced tumours were polyclonal. These findings, although not conclusive, suggest that an additional, rare change is needed for these tumours to develop. As we have identified the nonrandom loss of chromosome 15 as the possible additional change, the monoclonal nature of the *ras*/*myc* tumours is consistent with this hypothesis.

We cannot determine whether the loss of one chromosome 15 is an early event necessary for the genesis of tumours induced by v-Ha-*ras* plus v-*myc* or whether this change represents a cellular progression that occurs with a high frequency and is strongly selected. The latency periods of the tumours were unrelated to the nonrandom chromosome change, but the diploid polyoma-induced tumours grew more slowly and were more differentiated in appearance than the aneuploid *ras*/*myc* tumours. However, the fact that the polyoma-induced tumours did not show monosomy of chromosome 15 must indicate either that this change does not result in a selective advantage for the polyoma-transformed cells or that transfection with v-Ha-*ras* and v-*myc* DNAs induces this change more frequently than treatment with polyoma DNA. It is interesting to consider whether the loss of chromosome 15 is associated with any particular stage such as immortalization¹⁵ or tumorigenicity in the neoplastic progression of the Syrian hamster embryo cells. Karyotypic analyses of carcinogen-induced and spontaneously immortalized Syrian hamster embryo have not revealed any association with monosomy 15; in fact, a nonrandom gain of another chromosome (11) was observed¹⁶. However, transfection of immortalized cell lines with v-Ha-*ras* alone results in tumorigenic transformation⁷ and with some cell lines, a nonrandom loss of chromosome 15 was observed (unpublished data). Thus, the existing data indicate that the loss of chromosome 15 is closely associated with a later stage in the neoplastic progression of these cells.

Nonrandom chromosome loss has been observed in a variety of human and animal tumours and transformed cells^{1,2,17-19}. Pathak *et al.*²⁰ observed that 15 of 18 virus (simian adenovirus-7, chicken embryo lethal-orphan, herpes simplex and simian papova viruses)-transformed Syrian hamster cell lines were monosomic for chromosome 15. This result is similar to our findings with *ras*/*myc*-induced tumours.

We interpret our results to indicate that the loss of a specific chromosome results either in the loss of a cellular gene which effects a phenotypic change necessary or advantageous for neoplastic development or in the deletion of *trans*-acting cellular

sequences³ which regulate the expression of either the v-Ha-*ras* or v-*myc* genes. This additional change either occurred spontaneously in these cells or may have been induced by transfection with v-Ha-*ras* plus v-*myc* DNAs. Several authors have also proposed that the dosage of cellular factors regulates the expression of tumorigenicity in viral and chemically transformed cells²¹⁻²³. Chromosome loss from hybrids between normal and malignant cells^{24,25} results in the expression of tumorigenicity. In contrast to cell hybridization studies which suggest that tumorigenicity is a recessive phenotype^{24,25}, DNA transfection studies have indicated that tumorigenesis is due to dominantly acting genetic elements²⁶. Our findings that chromosome loss can occur after DNA transfection and tumour formation may help to resolve these seemingly disparate conclusions. The oncogene-transfection system may be useful for understanding chromosomal control in carcinogenesis and the role of cellular factors in the regulation of the expression of oncogenes and tumorigenicity.

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EGF receptor-associated DNA-nicking activity is due to a M_r -100,000 dissociable protein

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The receptor for epidermal growth factor (EGF) is a single-chain transmembrane polypeptide of relative molecular mass (M_r) 170,000 (170K) which has been implicated in the regulation of both normal and abnormal cell proliferation¹. It has an externally facing EGF-binding domain and a cytoplasmically facing tyrosine-specific protein kinase site. Although the receptor has been well characterized²⁻⁴, the mechanism by which it transmits the growth stimulatory signal from the plasma membrane to the nucleus is unclear. EGF binding to cells has been shown to enhance topoisomerase activity within the cells⁵. Topoisomerases catalyse the interconversion of topological isomers of DNA and thus may influence replication and transcription⁶. Mroczkowski *et al.*⁷ reported that purified EGF receptors of both human and murine origin can nick supercoiled double-stranded (ds) DNA in an ATP-dependent fashion, an activity related to those of topoisomerases⁶. Another related tyrosine kinase, pp60^{src}, has also been reported to have a similar DNA-nicking activity⁷. We have now characterized the EGF receptor-associated DNA-nicking activity by sucrose gradient centrifugation. Our results, presented here, indicate that the DNA-nicking activity is not intrinsic to the EGF receptor, but is found in a distinct molecular species.

The EGF receptor was solubilized and purified from plasma membranes of human A431 epidermoid carcinoma cells⁷⁻¹⁰. Receptor at various stages of purification (involving chromatography on EGF-AffiGel, wheat-germ lectin(WGA)-Sephacrose, and DNA-agarose) was tested for its ability to convert pBR322 DNA from form I to form II. Figure 1 shows the results obtained with equivalent amounts of receptor (measured by EGF-binding activity) at different stages of purification. As found previously, the DNA-nicking activity was ATP-dependent (Fig. 1a) and sensitive to KCl (Fig. 1b) and NaCl (not shown).

Form I DNA was converted to form II with no evidence of intermediate topoisomers, suggesting that the observed activity is an ATP-dependent nicking of supercoils rather than a classical topoisomerase activity. Further purification of the receptor on WGA-Sephacrose revealed that while over 80% of the EGF-binding activity was adsorbed to the column, most of the DNA-nicking activity was recovered in the unadsorbed fraction (not shown). The material eluted from the WGA-Sephacrose had markedly less DNA-nicking activity (per EGF-binding unit) than did the preparation loaded onto the column (Fig. 1). Nevertheless, the remaining DNA-nicking activity was still ATP-dependent and KCl-sensitive (Fig. 1b). These results demonstrate that the DNA-nicking activity found in the EGF-AffiGel-purified receptor preparation does not co-purify with the EGF receptor through the WGA-Sephacrose step.

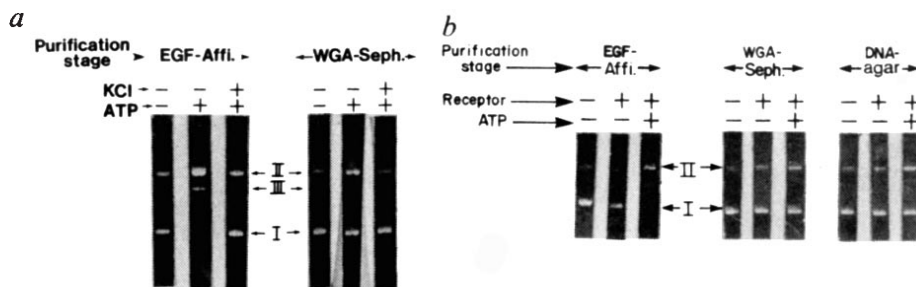
To confirm that EGF-binding and DNA-nicking activities are distinct, EGF-AffiGel-purified receptor was sedimented on a 5–20% sucrose gradient. Most (~80%) of both the EGF-binding activity and tyrosine autokinase activity co-sedimented at 7.7S (Fig. 2), with a minor peak of EGF-binding activity at 12S as a result of receptor dimerization, in agreement with our previous findings¹⁰. No DNA-nicking activity was seen at either 7.7S or 12S; a single peak of ATP-dependent DNA-nicking activity was seen at ~5.5S (M_r ~100,000), clearly separated from the EGF-binding and tyrosine kinase activities. Receptor further purified through WGA-Sephacrose and DNA-agarose gave identical results (not shown).

The results described above suggest that DNA-nicking activity is not an intrinsic activity of the 170K EGF receptor polypeptide. It is possible, however, that the DNA-nicking activity is the novel activity of a proteolytic cleavage product of the receptor. Trypsin treatment might thus be expected to enhance the DNA-nicking activity of receptor preparations; however no such increase was found (data not shown). Thus, evidence now available points to the conclusion that the observed DNA-nicking activity is due to an associated enzyme not related to the EGF receptor.

Note that the ratio of DNA-nicking protein to EGF-receptor protein is extremely small in our purified preparations. This is evident in the lack of detectable 100K protein (or proteins of lower M_r) in silver-stained gels (data not shown), and also in the microgram quantities of receptor protein needed for detection of DNA-nicking activity (Fig. 1).

Is the association between tyrosine kinases and ATP-dependent DNA-nicking proteins purely fortuitous? Topoisomerases are known to be bound to the 5' end of the nicked DNA through

Fig. 1 Separation of DNA-nicking activity and EGF-binding activity during WGA-Sephacrose chromatography. Supercoiled pBR322 DNA (0.2 µg) was incubated with an equivalent amount (0.5 pmol) of EGF receptor at the indicated stages of purification (amount of receptor was deduced by Scatchard analysis of ¹²⁵I-EGF-binding data). *a*, DNA-nicking activity in the presence and absence of receptor and ATP. *b*, Receptor-mediated DNA-nicking activity in the presence and absence of ATP and KCl. EGF-Affi., EGF-AffiGel; WGA-Seph., WGA-Sephacrose; DNA-agar., DNA-agarose.



Methods. Agarose gel electrophoresis was performed on 0.8% agarose gels in 40 mM Tris-acetate pH 7.9, 20 mM sodium acetate at room temperature for 18 h at 100 mA. The gels were stained with ethidium bromide (1 µg ml⁻¹), de-stained in water, and photographed under ultraviolet illumination. EGF receptor was solubilized from A431 plasma membranes and subjected to EGF-AffiGel chromatography^{3,8,9}. The EGF-AffiGel-purified receptor was subjected to further purification WGA-Sephacrose (Vector Laboratories) and ds DNA-agarose (Sigma)⁷. EGF-binding activity of the receptor preparation was determined as described elsewhere⁹. The amount of receptor present was deduced by Scatchard analysis of the ¹²⁵I-EGF-binding data, assuming a 1:1 complex of EGF and receptor. The conversion of supercoiled (form I) DNA to nicked DNA (form II) was carried out as described⁷ in 20 µl of 20 mM HEPES pH 7.4, 5% glycerol, 0.1% Triton X-100, 5 mM MgCl₂ containing 0.5 pmol of receptor (or no receptor), 5 mM ATP (or not ATP), 0.15 M KCl (or no KCl). After a 30-min incubation at 20 °C the reaction was stopped by adding 5 µl of 10% SDS. Alternatively, the DNA was precipitated by adding 3 µl of 3 M sodium acetate, and 70 µl of absolute ethanol at -20 °C. The products were analysed by agarose gel electrophoresis.

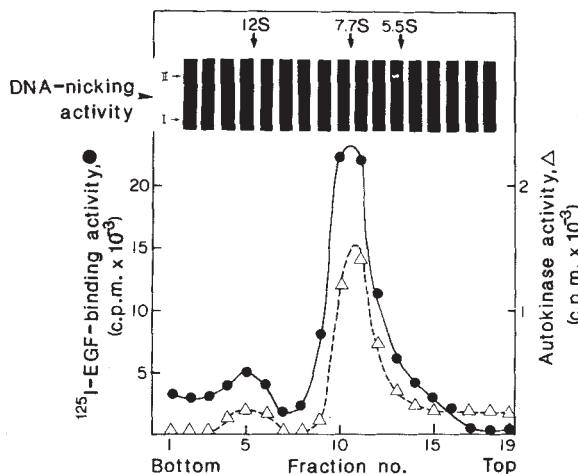


Fig. 2 Separation of the DNA-nicking activity and EGF-binding activity by sedimentation analysis. Sedimentation rates and M_s were estimated as described elsewhere^{10,14}. Approximately 10 pmol of EGF-AffiGel-purified receptor (in 100 μ l of 20 mM HEPES pH 7.4, 0.2% Triton X-100 and 3% glycerol) was centrifuged at 4°C for 9.5 h at 45,000 r.p.m. in a SW 50.1 swinging bucket rotor, through a 5–20% sucrose gradient (5 ml) in 20 mM HEPES pH 7.4, 0.2% Triton X-100 (ref. 10). The standard proteins (applied to separate tubes) were: bovine serum albumin (4.6S), human IgG (7S), and horse catalase (11S). At the end of the run, the tubes were punctured at the bottom and fractions were collected. Assays for standard proteins, EGF binding and autophosphorylation have been described elsewhere¹⁰. The EGF-binding values plotted (●) are specific binding data with 150 nM ¹²⁵I-EGF (150,000 c.p.m. ng⁻¹) which were obtained after subtraction of nonspecifically bound radioactivity (~4,000 c.p.m.). Receptor autophosphorylation (Δ) was quantified, after SDS-polyacrylamide gel electrophoresis⁸ of the gradient fractions reacted with 5 μ M [γ -³²P]ATP (100 c.p.m. fmol⁻¹), by counting gel slices containing the 150K/170K receptor band¹⁰. For assay of DNA-nicking activity, 100 μ l of each fraction was incubated with 10 μ l of a solution containing 50 mM MgCl₂, 40 mM ATP and 0.2 μ g of supercoiled pBR322 DNA. After incubation at 20°C for 30 min, the DNA was precipitated by adding 12 μ l of 3 M sodium acetate and 360 μ l of absolute ethanol at -20°C. The precipitate was subjected to agarose gel electrophoresis as described in Fig. 1 legend.

a phosphotyrosine linkage^{11,12}. The phosphorylation of this critical tyrosine by pp60^{src} (like the EGF receptor, a tyrosine-specific protein kinase) causes a drastic loss of topoisomerase activity¹³. Thus, it is probable that topoisomerase-related proteins associate with tyrosine kinases such as EGF receptor in an enzyme-substrate sense.

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Mitochondrial class II introns encode proteins related to the reverse transcriptases of retroviruses

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Organelle introns share several distinctive features that set them apart from their counterparts in nuclear-encoded pre-messenger RNAs (reviewed in ref. 1): (1) their termini do not obey the GU...AG rule; (2) the introns are 'structured' (members of the same family or 'class' can theoretically adopt very similar RNA secondary conformations^{1–3} and several of the postulated pairings have been confirmed by studies of splicing mutants and their revertants (see, for example, ref. 4); (3) many introns from both classes contain long open reading frames^{5–10}. We report here that the proteins potentially encoded by four class II introns are related to several RNA-dependent polymerases of viral and transposable element origins.

Four mitochondrial members of class II contain long open reading frames: introns a1 and a2 in the cytochrome oxidase subunit I gene of the yeast *Saccharomyces cerevisiae*⁵; an intron in the corresponding gene of the filamentous fungus *Podospira anserina*¹¹; and the single intron in the apocytochrome *b* gene of *Schizosaccharomyces pombe*¹². The sequences of these introns are sufficiently similar to be aligned over most of their length. Recently, the protein potentially encoded by a mitochondrial plasmid of the Mauriceville-1c strain of *Neurospora crassa*¹³, another filamentous fungus, was shown to constitute the fifth known member of this family¹². The relationship is rather distant, as alignment of the plasmid-encoded protein with the four intronic ones is only possible over short amino-acid stretches (blocks I–VII in Fig. 1), all located in a section that does not span more than one-third of the estimated length of the molecules.

The possibility of the Mauriceville plasmid being related to transposable elements was suggested by Nargang *et al.*¹³. To test this hypothesis, we compared the seven amino-acid stretches conserved in the plasmid putative protein product and its intronic relatives with those characteristic of a family of RNA-dependent polymerases, which includes the products of the *pol* genes of retroviruses and a *copia*-like element as well as the related proteins encoded by cauliflower mosaic and hepatitis B viruses^{14–16} (segments III, IV and V in Fig. 1 correspond to segments I, II and III, respectively, of ref. 15).

As shown in Fig. 1, each of the mitochondrial conserved segments has a counterpart in viral sequences. Moreover, not only is the order of conserved blocks the same in the two sets, but distances (in amino acids) from one block to the next are rather similar, with two exceptions: the four intronic proteins (but not that encoded by the Mauriceville plasmid) have large insertions of poorly conserved sequence between segments IV and V; the Mauriceville plasmid sequence (and also that of the hepatitis B viral protein) possesses a large insertion between segments III and IV. Note also that 9 of the 10 residues found by Toh *et al.*¹⁴ to be invariant in the set of RNA-dependent polymerases they compared could each be identified in at least 4 of the 5 mitochondrial sequences.

The section of the *pol* gene that has a counterpart in the reading frames of class II introns is thought to be responsible for the polymerase activity (see ref. 14). Two lines of evidence have already indicated the presence of a reverse transcription

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Fig. 1 Alignment of similar segments in mitochondrial intron- and plasmid-encoded proteins and RNA-dependent polymerases. Amino-acid sequences were deduced from nucleotide sequences. Mitochondrial sequences are those of the proteins potentially encoded by introns S.c.a1 and S.c.a2 of *S. cerevisiae*⁵, P.a.a1 of *P. anserina*¹¹ and S.p.b1 from *S. pombe*¹², together with that of the putative protein product of a *N. crassa* plasmid¹³ (N.c.mau.). Retroviral sequences are those of the *pol* genes of Rous sarcoma virus³² (RSV), human adult T-cell leukaemia types I and II viruses^{33,34} (HTLV-I and -III) and Moloney murine leukaemia virus (MoMLV)³⁵. Other sequences are those of the putative polymerase of cauliflower mosaic virus³⁶ (CaMV), the product of the *pol*-like gene 17.6 (ref. 16), a transposable element and a putative polymerase encoded by hepatitis B virus³⁷ (HBV). Amino acids that are conserved in at least four sequences belonging to two different sets are boxed (only the most common amino acid at any single site is boxed). Note that many substitutions, even at the less-conserved sites, are conservative. Arrowheads point to the location of 9 of the 10 amino acids reported as invariant by Toh *et al.*¹⁴.

		I		II		III		
Mitochondrial	S.c.a1	MVN-PKPKGH--RPLSV	32 aa	YNN-SQITAIW	11 aa	PIEMDLKCFDTISD	38 aa	
	S.c.a2	RVGI-PKLSGCF--RPLSV	32 aa	PAL-SQITALL	11 aa	PIKVDLKKCFDTLPHN	38 aa	
	P.a.a1	RVVL-PKPKGH--RPLGI	32 aa	PKR-GCHSALA	10 aa	PIEGDLKCFDTLPHH	37 aa	
	S.p.b1	RVLI-PKASGK--RPLTI	32 aa	PKR-SHGSALR	11 aa	WIEGDLKCFDTLPHH	37 aa	
	N.c.mau	RVYI-PKANGK--RPLGV	31 aa	PKR-GVFTAMR	10 aa	IVFDLKNFTFSTNLA	82 aa	
Retroviral	RSV	VFPV-RKASQSY--RPLHD	15 aa	QGA-PVRSALP	4 aa	LMVLDLKKCFDTLPIA	25 aa	
	HTLV-I	VFPV-KKANGTW--RPLHD	15 aa	PGP-PDLGSLP	5 aa	LQTDLDKDAFFGLPLP	25 aa	
	HTLV-III	VFAKKKPSITW--RPLVD	15 aa	LCI-PHPAGLK	4 aa	VTVDVGVDAFFSPLPD	25 aa	
	MoMLV	LLPV-KK-PGTDNDY--RPLVD	15 aa	PKPYNLGSLP	5 aa	YTVLDLKKDAFFGLPLP	24 aa	
Other	CaMV	PKRKK--RNVVN	15 aa	PNK-DELLTI	5 aa	PSSFDKRSFQVLLD	18 aa	
	17.6	D-ASGKQKFIIVD	15 aa	PNH-DELLCKL	5 aa	HTTIDLKKDFFHGLPLP	18 aa	
	HBV			PNL-QSITML	5 aa	WLSLGLVSAFFHGLPLP	72 aa	
		IV		V		VI	VII	
Mitochondrial	S.c.a1	LGLPQGSLSPLIC	70 aa	YVRVADDTLIG	22 aa	GLTNCEK	11 aa	FLGYND
	S.c.a2	LGLPQGSVVSPLIC	73 aa	FVRVADDTLIG	23 aa	GMSINHDKS	10 aa	FLGYDV
	P.a.a1	IGVPQGSIASPLIS	89 aa	YVRVADDTLIG	23 aa	KHELSEKTK	11 aa	FLGTEI
	S.p.b1	VGPVQGSIVSPLIA	75 aa	YVRVADDTLVA	22 aa	GLTVSRKTK	11 aa	FLGTNI
	N.c.mau.	NGVPQGSISGLIA	13 aa	LIMVADDTLIG	14 aa	GVVQKAKS	14 aa	FLGTEF
Retroviral	RSV	KVLPQGSITSPITIC	19 aa	MLRVADDTLVA	21 aa	GLTVSRKTK	7 aa	FLGYKL
	HTLV-I	KVLPQGSITSPITIC	19 aa	ILQVADDTLVA	21 aa	GLTVSRKTK	8 aa	FLGYKL
	HTLV-III	NVLPQGSITSPITIC	19 aa	IYQVADDTLVA	22 aa	GLTVSRKTK	7 aa	FLGYKL
	MoMLV	TRLPQGSITSPITIC	19 aa	LLQVADDTLVA	21 aa	GLTVSRKTK	8 aa	FLGYKL
Other	CaMV	NVVPQGSISPLIF	14 aa	CCVVDVDTLVA	21 aa	GLTVSRKTK	8 aa	FLGYKL
	17.6	LRMPLKKNAPATIF	15 aa	CLVVDVDTLVA	21 aa	MLKLQDKIC	8 aa	FLGYKL
	HBV	RKIDMVGSLSPITIC	19 aa	AFSDVVDVDTLVA	21 aa	GLTVSRKTK	8 aa	FLGYKL

activity in fungal mitochondria. First, a number of *S. cerevisiae* intronic mutants revert preferentially by clean excision of the affected intron and often of one or more of its immediate neighbours¹⁷ (genetic arguments even suggested that intron-encoded proteins are involved in the process). Second, the most common form of senescence in *P. anserina* is associated with proliferation of a mitochondrial plasmid whose sequence coincides precisely with that of an intron in the cytochrome oxidase subunit I gene of this fungus¹¹. This intron is one of the four class II members discussed here.

On the other hand, the phenotypes associated with mutations in the reading frame of intron a1 of *S. cerevisiae* point to an activity other than reverse transcription. The reading frame of intron a1 continues that of the upstream exon⁵. This is the most commonly encountered situation in organelle introns and has

led to the view that their primary translation products are chimaeric proteins^{7,18,19}, another point of convergence with retroviruses and transposable elements²⁰⁻²². But although the level of expression of *pol* gene products seems to be adjusted at the translation stage (see, for example, ref. 22), synthesis of intron-encoded proteins has been postulated to be regulated at the post-transcriptional level. This is termed the 'maturase'^{7,18} or 'splicing'¹⁹ model, according to which chimaeric protein translated from a pre-mRNA molecule (or its proteolytic derivatives, see Fig. 2) contributes to the destruction of its own mRNA by participating in the splicing process (in this model it is assumed that the excised form of the intron, which, in the case of a1, is a stable circular RNA²³, does not give rise to a functional translation product). Several *trans*-recessive splicing-deficient mutations have been identified that map within the open reading frame of intron a1 and block its excision²⁴.

However, that the protein encoded by intron a1 should be involved in excision of the intron does not contradict the fact that part of it is related to reverse transcriptases. Several structured introns catalyse their own excision from primary transcripts without the need for a protein helper^{25,26} (the best-known example is provided by the *Tetrahymena* ribosomal intron, one of the few nucleus encoded class I introns^{1,27,28}). Thus, as far as splicing is concerned, maturases seem to be accessory devices and maturase activity could have evolved from intron-associated proteins as a form of self-regulation. On the other hand, possession of a specialized reverse transcriptase, although of no normal use in organelles, could be of decisive advantage in the maintenance and propagation of structured introns in the genomes of organelles. Thus, it will be of interest to determine whether the proteins encoded by class II introns have a DNA endonuclease activity similar to that believed to be involved in integration of retroviral DNA into the host genome²⁹⁻³¹, which is encoded by the 3' section of *pol* genes (see Fig. 2).

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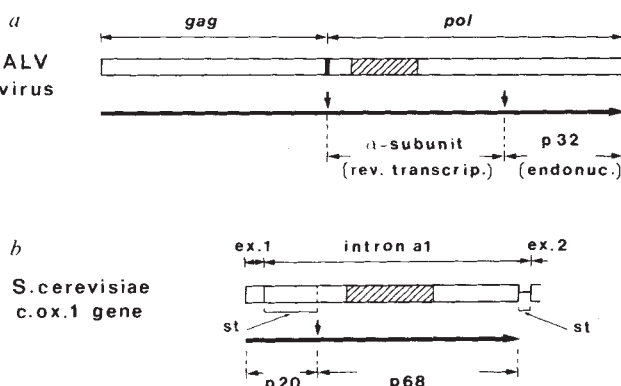


Fig. 2 Organization and expression of the *pol* gene of avian sarcoma-leukosis virus (ALV) compared with those of intron a1 in the cytochrome oxidase subunit I gene of *S. cerevisiae*. *a*, The gene and its products, redrawn from ref. 38; *b*, intron a1, redrawn from ref. 24. Homologous sections in the two genes (see Fig. 1) are hatched. Primary translation products are sketched as thick lines with an arrow-shaped extremity that indicates the direction of translation. Vertical arrows point to proven or presumed sites of proteolytic cleavage; st, sections of intron a1 that participate in class II secondary structure ('core' of the intron, see ref. 2). In the case of intron a1, the active product has been proposed to be p68 (ref. 24), a polypeptide which seems to correspond to most or all of that part of the intronic reading frame that is looped out of the structured core of the intron by long-range potential base pairings, also well conserved in evolution^{2,12}.

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Haemophilia B caused by a point mutation in a donor splice junction of the human factor IX gene

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Haemophilia B (Christmas disease) is an inherited, recessive, sex-linked, haemorrhagic condition caused by a defect in the intrinsic clotting factor IX. This disease occurs in males at a frequency of ~1 in 30,000. Patients differ in the severity of their clinical symptoms, and variation in the clotting activity and in the concentration of factor IX antigen in their plasma has been demonstrated¹. There is probably heterogeneity in the molecular defects of the factor IX gene causing the disease. Here we study a severely affected, antigen-negative patient, and show that the only significant sequence difference from the normal factor IX gene is a point mutation changing the obligatory GT to a TT within the donor splice junction of exon f. We infer that this change is the cause of the disease in this individual. In addition, we have used oligodeoxynucleotide probes specific for this mutation to demonstrate the feasibility of carrier detection and prenatal diagnosis for relatives of the patient.

The characterization of the normal factor IX gene² made it possible to characterize haemophilia B at a genetic level. Approximately 40% of patients have a normal concentration of factor IX antigen (IXAg) in their plasma³ and probably have point mutations in the coding sequence giving rise to single

amino-acid changes such as that found in factor IX_{Chapel Hill} (ref. 4). A few patients (1%) produce antibodies to the injected factor IX used in replacement therapy and here the defect appears, in most cases, to be large deletions in the factor IX gene⁵. The remaining 60% of patients have either markedly reduced or undetectable levels of IXAg and can be described as cross-reactive-material negative (CRM negative)¹. It is plausible that the defect in this group of patients could be either mutations resulting in a reduction in the amount of factor IX messenger RNA, or chain termination mutations causing the production of truncated and presumably unstable factor IX protein.

The patient (D.B.) chosen to test this hypothesis falls into the severe, CRM-negative class, having 0.3% factor IXAg⁶ (assayed by radioimmunoassay⁷) and <0.5% factor IX clotting activity⁶ (assayed by two-stage thromboplastin generation assay⁸). A genomic cosmid library was prepared from DNA isolated from the patient's peripheral blood lymphocytes and cloned in the cosmid vector pTM (ref. 9). Four recombinant clones containing factor IX sequences were isolated and mapped as before² (Fig. 1). From these clones, all eight exon regions were separately subcloned and sequenced¹³, except for that part of exon h corresponding to the 3' noncoding section of the mRNA. The sequences determined totalled 5,200 bases, covering the presumptive promoter, the 5' noncoding region of the mRNA, all protein-coding sequences and the polyadenylation signal AATAAA.

The sequence was found to be identical to the previously published normal factor IX gene² with 14 exceptions. A G→T change was found at position 21,165, within the obligatory GT of the donor splice site at the 3' site of exon f (Fig. 2), which changes an *RsaI* site in the normal sequence to a *MaeIII* site

Fig. 1 Line diagram of the positions of clones isolated from patient D.B. in relation to the organization of the factor IX gene. *a*, The position of the eight exons a-h; *b*, the position of cosmid clones isolated from patient D.B.; *c*, the subclones generated for sequencing.

Methods. DNA was isolated¹⁰ from peripheral blood lymphocytes from 100 ml of whole blood. It was partially restricted with *MboI* and size fractionated on a 1-5 M NaCl gradient⁹. DNA in the size range 35-45 kb was pooled, ligated into the cosmid vector pTM⁹, packaged into bacteriophage λ heads¹⁰ and allowed to infect *Escherichia coli* ED 8767 (ref. 11). 1.5×10^6 clones were screened by colony hybridization¹² using previously isolated factor IX probes XI, IV and cVII (ref. 2). Positive clones were mapped by digestion with restriction enzymes to the positions shown by comparison with maps of previously characterized genomic clones. Clone pDB3 contains approximately 15 kb of the factor IX gene ligated to approximately 20 kb from elsewhere in the genome as a result of a cloning artefact. Subclones constructed for sequencing were derived by restriction digestion of the appropriate cosmid clone and isolation of the required fragments, which were "filled in" with deoxynucleoside triphosphates using the Klenow fragment of *E. coli* DNA polymerase¹⁰ and blunt-end ligated into the vector pATX (C. Naeve, unpublished) except for subclones XIV and VI. These were ligated directly into the *EcoRI* site of pBR322. The restriction sites at the ends of each subclone are: B, *BglII*; E, *EcoRI*; H, *HindIII*; L, *BglI*; P, *PvuII*. Subclones are numbered as before².

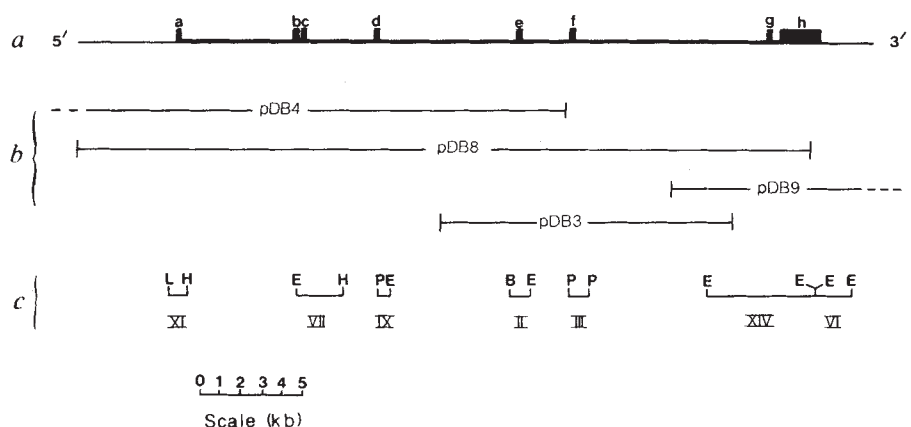
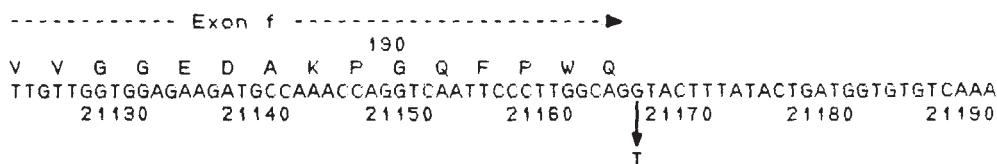


Fig. 2 The sequence surrounding the 3' end of exon f of the normal factor IX gene showing the position of the G→T mutation found in the donor splice site in patient D.B. The other sequence changes found in D.B. were G→C at position 6,928 at a previously discovered polymorphic *XmnI* site¹⁴, T→C at position 34,037 and A→G at an unmapped position not more than 1.4 kb from the 3' end of the gene. Nine other differences reflected sequence errors in the normal factor IX gene sequence and corrections to Fig. 4 of ref. 2 are as follows: 138=G, 139=A, 309=T, 310=C, 319=A, delete residues 6,050, 34,026 to 34,031 and 34,093, insert T between residues 34,107 and 34,108 and insert T between residues 34,170 and 34,171. (Fig. 2 of ref. 2 should also be corrected at the three relevant positions.)



in the patient. A G→C change was found at position 6,928 resulting in the loss of an *XmnI* site previously shown to be polymorphic in normal populations¹⁴ (see Fig. 2 legend). Two further differences were found 3' to the factor IX gene (see Fig. 2 legend), and these may also be polymorphic but do not result in the change of any restriction enzyme sites. The 10 remaining differences were all caused by errors in the normal factor IX sequence² (see Fig. 2 legend).

To test the feasibility of directly assaying the molecular defect in total DNA isolated from peripheral blood lymphocytes, we synthesized oligodeoxynucleotide probes¹⁵, designed according to the principles developed to detect point mutations in globin genes¹⁶, to analyse this mutation in the patient and his mother (Fig. 3). Two 19-base long oligodeoxynucleotides (probes 1 and 2; see Fig. 3 legend) were used to hybridize to uncloned and cloned sequences under conditions in which only exactly matching sequences hybridized¹⁶. Probe 1 (exactly complementary to the normal factor IX sequence) hybridizes to a 1.1-kilobase (kb) *PvuII* restriction fragment² in digests of DNA isolated from a normal male, from a normal female, from a cell line with 4X chromosomes (GM1416) and from the patient's mother (M.B.) (Fig. 3B a-c, e), as well as in *PvuII*-restricted cloned normal factor IX DNA (Fig. 3B g). Thus, probe 1 hybridizes to the correctly sized restriction fragment and with the intensity expected

for an X chromosomal sequence (that is, with a ratio of intensities of about 1:2:4 on 1X, 2X and 4X genomic DNA). In addition, probe 1 fails to hybridize to the mutant sequence either in uncloned or cloned DNA (Fig. 3B d, f).

Probe 2 (exactly complementary to the mutated sequence in the patient) hybridizes to the same restriction fragment only in *PvuII* digests of DNA isolated from the patient, his mother and the cloned mutant factor IX gene (Fig. 3B k-m) and fails to hybridize to the normal sequence (Fig. 3B h-j, n). Thus, these probes differentiate specifically between the normal and mutant sequences and in addition confirm that the base change characterized is not a cloning artefact. Both probes also hybridize to the DNA isolated from the patient's mother, in agreement with her obligatory carrier status (Fig. 3). If DNA were available from II₅ and II₆, it would be possible to determine their carrier status. If they are carriers, both probes 1 and 2 would hybridize, whereas if not, only probe 1 would hybridize. It would also have been possible to use digestion with *RsaI* and Southern blot analysis to assay the molecular defect directly. This should show a patient-specific fragment of 1.3 kilobases (kb) when probed with probe III², compared with a normal fragment of 1.1 kb.

We then analysed the DNA from nine other unrelated CRM-negative patients whose factor IX antigen levels ranged from 0 to 6% of normal in a radioimmune assay^{6,7}. Under identical hybridization conditions to those used above, all hybridized to probe 1 (Fig. 4) and failed to hybridize to probe 2 (results not shown), indicating that they had the normal sequence at this position. Presumably the defect in these patients occurred elsewhere in the factor IX gene, either in other splice donor or acceptor sequences or other sequences essential for normal gene expression, as has been found to be the case in α and β thalassaemias¹⁷.

We have shown that a point mutation (G→T) within the obligatory GT of a donor splice junction is the only significant sequence change in the factor IX gene from patient D.B. It is well established that this dinucleotide is highly conserved¹⁸ and essential for the normal splicing of pre-mRNA to mRNA so that any alteration of this sequence would be expected to perturb this process. Perhaps the most convincing supporting evidence is that mutations in the GT of donor splice sequences in the

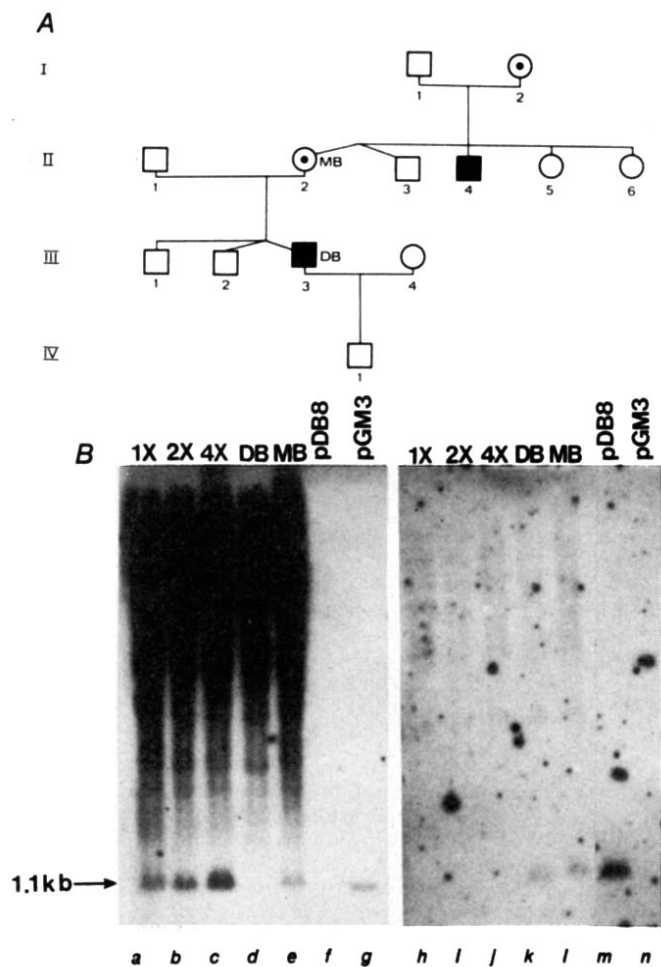


Fig. 3 A, family tree of the D.B. family showing the inheritance of haemophilia B. ■, Affected male; ○, obligatory carrier. The status of II₅ and II₆ is unknown. B, oligonucleotide hybridization studies. Two oligonucleotide probes were synthesized¹⁵, being complementary to residues 21,157–21,175. Probe 1 is the normal sequence 5' GTATAAAGTACCTGCCAAG 3' and probe 2 is the mutated sequence 5' GTATAAAGTAACTGCCAAG 3', differing from probe 1 at position 11. These were used for hybridization studies to genomic and cloned factor IX sequences as shown. a–e, h–l contain 15 µg of the indicated genomic DNA; f, m, 0.5 ng and 10 ng respectively of pDB8; g, n, 0.5 ng and 10 ng respectively of pGM3 (a cosmid clone, containing the 5' half of the normal factor IX gene, D.J.G.R., unpublished). DNA samples were digested to completion with *PvuII* and electrophoresed in a 20-cm 0.7% agarose gel in 90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, pH 8.3. The gel was then treated as described by Conner *et al.*¹⁶. 50 ng of each oligonucleotide was labelled with [γ -³²P]ATP using T4 polynucleotide kinase (Amersham) to a specific activity of 10⁹ d.p.m. µg⁻¹ and hybridized to the dried gel for 16 h at 42 °C. The gels were then washed as described, with a final temperature of 60 °C in 6×SSC¹⁶.

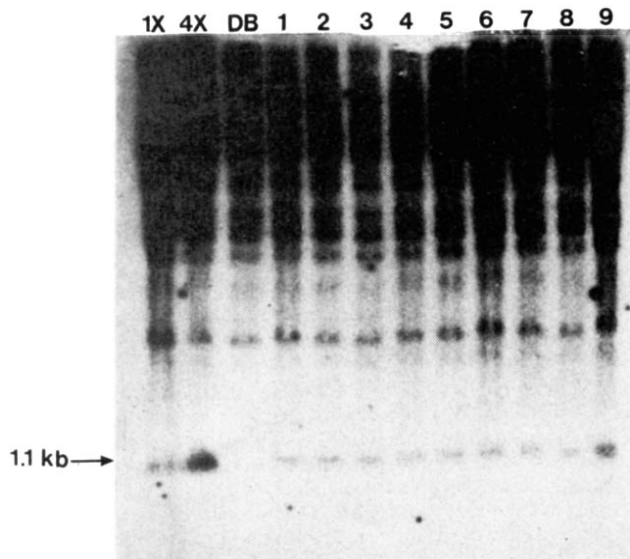


Fig. 4 Hybridization of ^{32}P -labelled probe 1 to DNA (15 μg per track) from nine CRM-negative haemophilia B patients from unrelated kindreds⁶ (tracks 1-9). The samples were digested, electrophoresed in 0.7% agarose, hybridized and washed as described in Fig. 3. 1X and 4X DNA samples (15 μg) were included as positive controls; DNA (15 μg) from the patient D.B. was used as a negative control. Hybridization of probe 2 under identical conditions showed a positive signal only on the track containing DNA from D.B. (data not shown).

β -globin gene are known to cause β^0 thalassaemias with no production of cytoplasmic β -globin mRNA¹⁷. We have been unable to study the effect of this mutation on the *in vivo* splicing of the factor IX pre-mRNA in our patient because of the difficulty of obtaining liver biopsy samples, which are the source of the hepatocytes that express factor IX. However, in theory, if the incompletely spliced mRNA were translated and the protein processed normally, a truncated protein of 217 residues would be produced which extends 22 amino-acid residues from exon f and terminates at TGA at nucleotide 21,231 (ref. 2). In practice, there is doubt that this occurs because virtually no factor IX antigen is detectable in plasma by radioimmunoassay.

It has been possible in both α and β thalassaemias to use oligodeoxynucleotide probes to detect single base changes in genomic DNA. Here we have shown that this approach can be successfully applied to haemophilia B, both to detect a single base change in the patient and to detect the presence of the mutant allele in a heterozygote. This should allow the use of mutation-specific probes to perform prenatal and carrier diagnosis of haemophilia B in families carrying mutations that have been characterized at the molecular level. In addition, using the same techniques, it should be possible to analyse naturally occurring sequence differences such as those characterized here 3' to the factor IX gene, which do not alter restriction enzyme cleavage sites in genomic DNA, to determine whether they are polymorphic in the normal population. If this is the case, then their use in linkage analysis should extend the range of carrier and prenatal diagnostic facility currently available for haemophilia B^{14,19}.

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Attachment of transported vesicles to microtubules in axoplasm is facilitated by AMP-PNP

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Axoplasm extruded from the squid giant axon has been used to analyse the molecular mechanisms of intracellular vesicle transport^{1,2}. Using video-enhanced light microscopy, vesicle transport can be observed directly on individual microtubules at the edge of the axoplasm²⁻⁴. Here we report that AMP-PNP (adenyl-5'-yl imidodiphosphate), a non-hydrolysable analogue of ATP, reversibly inhibited vesicle transport. Moreover, vesicles in solution attach to the microtubules and form relatively stable complexes. AMP-PNP may produce this effect by binding to an ATP-binding site on the transport machinery, thereby stabilizing the motility complex that is normally formed by a transported vesicle, an ATPase and a microtubule. The effects of AMP-PNP on the vesicle transport system indicate that the enzymatic machinery of this system differs significantly from that of the actomyosin system or the dynein-microtubule system.

Using the extruded axoplasm model, we have begun to explore the following question: is the molecular mechanism of vesicle transport similar to the two well-characterized models of cell motility in metazoa, the actomyosin-based system in muscle^{5,6} and the dynein-based system in the axonemes of flagella and cilia^{7,8}? One basic property of both these systems is their response to changes in the concentration of ATP. When ATP is absent, myosin and dynein form stable (rigor) complexes with their motile partner (actin and the B subfibril, respectively)⁵⁻⁸. By contrast, when ATP is added to a muscle or an axoneme in rigor, the rigor complexes rapidly dissociate and the ATP is hydrolysed⁶⁻¹⁰.

Non-hydrolysable analogues of ATP, such as AMP-PNP, can be used selectively to block events in the cycle of motility which are dependent on the hydrolysis of ATP¹¹. In muscles and axonemes, AMP-PNP stabilizes those transient intermediates that occur in the motility cycle just before ATP hydrolysis⁶⁻¹⁴. AMP-PNP acts by binding specifically to the ATP binding site on myosin and dynein; and, like ATP, it tends to dissociate the actomyosin and dynein-B subfibril complexes *in vitro*¹⁰⁻¹⁴. However, myosin and dynein have a lower affinity for AMP-PNP than for ATP, and AMP-PNP is less effective than ATP at dissociating the rigor complexes¹⁰⁻¹⁴.

We have used AMP-PNP to obtain information about the transient intermediates that are formed before ATP hydrolysis in the motility cycle of vesicle transport. Perfusion of axoplasm with a physiological buffer (see Fig. 1 legend) containing 0.5-

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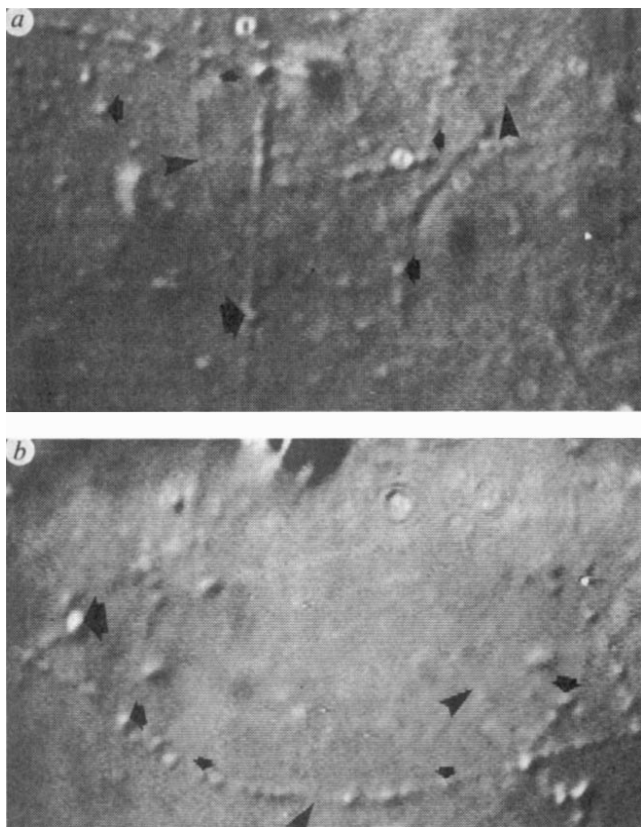


Fig. 1 Microtubules at the periphery of the axoplasm (arrow-heads). *a*, Isolated microtubules in buffer X without AMP-PNP. Vesicles in solution frequently attach and translocate along the microtubules. Arrows, examples of vesicles moving along microtubules. *b*, Microtubules in the presence of 5 mM AMP-PNP (lithium salt). In the presence of AMP-PNP, vesicles attach to the microtubules and remain stationary, producing the appearance of a 'string of pearls'. Small, medium and large vesicles are indicated by different sized arrows. In parallel experiments, Li^+ at concentrations of 1–5 mM had no effect.

Methods. In all the experiments described here, axoplasm was obtained from the medial third-order giant fibres of squid (*Loligo pealei*). Briefly, the giant fibres were tied off at each end and removed from the animal^{2,20}. After removing excess connective tissue, the fibre was rinsed in buffer X (350 mM potassium aspartate, 130 mM taurine, 70 mM betaine, 50 mM glycine, 20 mM HEPES, 12.9 mM MgCl_2 , 10 mM EGTA, 3 mM CaCl_2 , 1 mM glucose, 1 mM ATP, pH adjusted to 7.2 with KOH), blotted dry and the proximal end cut². A cylinder of axoplasm was extruded onto a coverslip, which formed the lower surface of the observation chamber². To form the side walls of the chamber, two thin spacers were placed on either side of the axoplasm. A space of ~1 mm was left between each spacer and the axoplasm, and a coverslip with spacers was gently placed on top of the axoplasm to complete the chamber. All the experimental observations began with a scan of the axoplasm to ensure that the preparation was active; then 10 μl buffer X containing 0–1 mM ATP and 0–10 mM AMP-PNP was perfused through the chamber. Movement was observed in the centre and at the periphery for 30 min or until no movement was observed. Video-microscopy was carried out using AVEC-DIC (video-enhanced differential interference contrast) microscopy using a Zeiss Axiomat microscope and Hamamatsu video equipment^{1,3}.

10 mM AMP-PNP immediately blocked the movement of vesicles on microtubules at the periphery of the axoplasm (Fig. 1). Below 0.5 mM AMP-PNP, movement was not effectively blocked.

At AMP-PNP concentrations which blocked transport, free vesicles in the solution attach normally to the peripheral microtubules but do not translocate. After 3–5 min, so many vesicles had attached to the microtubules and then remained stationary that the microtubules had the appearance of a 'string of pearls' (Fig. 1*b*).

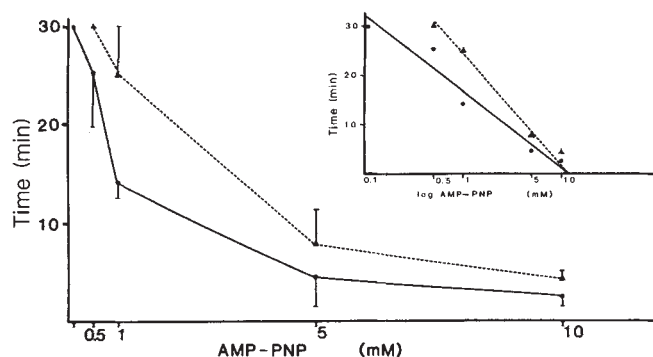


Fig. 2 Effects of ATP and AMP-PNP in the presence and absence of exogenous ATP. Axoplasm was extruded as described in Fig. 1 legend. A known volume of buffer, which contained either AMP-PNP alone (circles) or AMP-PNP with 1 mM ATP added (triangles and dashed line), was perfused into the chamber. The ratio of the buffer volume to axoplasm volume was ~5:1. Vesicle movement was observed in the centre of the axoplasm and recorded every 5 min until either >95% of the vesicles had stopped moving or 30 min had elapsed. The results are plotted as a function of increasing concentration of AMP-PNP against time taken for inhibition of vesicle movement. All experiments were done in replicate with $n = 2-4$ for each condition; vertical bars indicate s.e.m. The difference between the time to inhibition with AMP-PNP alone and with AMP-PNP+1 mM ATP is significant for all concentrations tested at $P > 0.01$. The inset shows the same data plotted against the log of the concentration of AMP-PNP. Lines on the inset were fitted by the linear regression method and each line had a regression coefficient of $r = 0.94$. These dose-response experiments indicate that it takes ~25 min for the lowest effective concentrations of AMP-PNP (0.5 mM) to inhibit reproducibly vesicle movement in the centre of the axoplasm. Presumably, this represents the interval required for the concentrations of AMP-PNP and ATP to equilibrate between the axoplasm and the external buffer because vesicle movement stops immediately at the periphery of the axoplasm.

In the central axoplasm, vesicle movement was also inhibited by AMP-PNP². At 10 mM exogenous AMP-PNP, levels of AMP-PNP in the centre of the axoplasm reached a sufficient concentration to inhibit vesicle movement after only 2 min. At lower concentrations complete arrest of vesicle movement in the centre of the axoplasm was delayed by the time that it takes for sufficient AMP-PNP to diffuse from the external buffer into the axoplasm (Fig. 2).

AMP-PNP inhibited vesicle movement at concentrations as low as 0.5 mM, which is similar to the ATP concentration in the axoplasm. Normally, axoplasm contains ~1 mM endogenous ATP but when the axoplasm and buffer in the perfusion chamber equilibrate, the final concentration of ATP is ~0.2 mM. This concentration of ATP is sufficient to maintain normal vesicle transport for the duration of the experiment. Thus, AMP-PNP completely inhibited particle movement in the presence of ATP at a concentration that normally maintains motility. By contrast, AMP-PNP does not completely inhibit motility in muscle and axonemes when the concentration of ATP is sufficient to maintain motility.

AMP-PNP may block vesicle transport by effectively competing with ATP for the nucleotide binding site or sites in the vesicle transport machinery. To test this hypothesis, we examined the effects of adding exogenous ATP on the inhibition of vesicle movement by AMP-PNP (Fig. 2). We found that 1 mM ATP significantly delayed the inhibitory effect of AMP-PNP at all concentrations and completely blocked the inhibitory effects of 0.5 mM AMP-PNP. Moreover, the inhibitory effect of AMP-PNP on vesicle transport could be reversed by perfusion with excess ATP.

In the ATP reversal experiments, the observation chamber was first perfused with 1 mM AMP-PNP to inhibit vesicle movement and then perfused twice with 10 μl buffer containing 10 mM ATP. Vesicle movement resumed at the periphery within

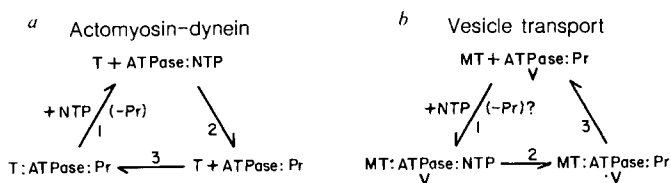


Fig. 3 Schematic enzyme mechanisms for intracellular motility cycles⁷⁻⁹ comparing the actomyosin and dynein ATPase cycles (a) with the vesicle transport cycle (b). a, 'ATPase' refers to either myosin or dynein and T refers to the translocatable partners (that is, filamentous actin or the B subfibril). NTP refers to nucleotide (for example, ATP or AMP-PNP) and Pr refers to the products of ATP hydrolysis. The drive stroke for myosin and dynein occurs after step 3. b, 'ATPase' refers to the putative ATPase in the crossbridge that mediates the attachment of the translocatable vesicles (V) to the microtubules (MT)¹⁵. We propose that ATP modulates the affinity of the ATPase-vesicle complex for the substrate microtubule. This hypothesis is supported by the observation that vesicles prepared from squid axoplasm retain their motile ATPase and can translocate along flagellar microtubules¹⁷. For simplicity the scheme is drawn as if the vesicle transport ATPase has a stable association with the vesicle. However, the ATPase can apparently dissociate from the vesicle. The step at which products (Pr) are released remains to be determined in both (a) and (b). Note that the effect of the binding of nucleotide (NTP) in the actomyosin and dynein cycles (a) is the opposite of its effect in the vesicle-transport cycle (b).

1-2 min of ATP addition, and vesicles began to move in the centre of the axoplasm 10 min after ATP addition.

Based on these results, we propose that AMP-PNP operates by competitively attaching to a critical ATP binding site in the motility machinery of vesicle transport. Miller and Lasek¹⁵ have recently proposed that the critical ATP binding site is an ATPase that is located on the 16-18-nm-long crossbridges that attach the transported vesicle to its substrate microtubule. Thus, like the actomyosin system and the dynein-microtubule system, motility in the vesicle transport system may be based on an ATP-dependent crossbridge cycle.

ATP has two important functions in the crossbridge cycle of muscles and axonemes⁷⁻⁹; it provides energy for movement and reduces the affinity of the crossbridge (that is, myosin or dynein) for its motile partner (that is, actin or tubulin) (Fig. 3a). In muscle, the biochemical cycle producing movement begins with the binding of ATP to the stable actin-myosin (rigor) complex⁹ which induces a rapid release of the myosin crossbridge from actin. Myosin hydrolyses the ATP, producing an activated ADP-myosin complex that has a much higher affinity for actin than the ATP-myosin complex. When the activated ADP-myosin complex rebinds to actin, it releases its stored energy in the motile power stroke. A similar scheme has been proposed for axonemes^{7,8}.

The effects of AMP-PNP on vesicle transport suggest that the enzymatic cycle of vesicle transport differs in at least two important ways from the cycle proposed for muscle and axonemes. First, dynein and myosin ATPases are weakly inhibited by AMP-PNP, that is, AMP-PNP will not produce complete inhibition of the biochemical cycle at concentrations of ATP that support motility^{6,14}. By contrast, the vesicle transport ATPase is completely inhibited by AMP-PNP even in the presence of concentrations of ATP that readily support vesicle transport. Second, in both muscles and axonemes, nucleotide binding promotes the dissociation of the motility complex (Fig. 3a, step 1)^{6,10,13}. In contrast, AMP-PNP facilitates a stable association of vesicles with microtubules. This suggests that nucleotide binding (for example, ATP or AMP-PNP) to the vesicle-transport ATPase promotes the formation of the basic motility complex (the vesicle-crossbridge-microtubule complex; Fig. 3b, step 1), which is the opposite of the effect of nucleotide on myosin and dynein (Fig. 3a).

The scheme proposed for the vesicle-transport cycle in Fig. 3b is further supported by observations of vesicle movement in ATP-depleted axoplasm. When ATP is eliminated from squid axoplasm by conversion to AMP and pyrophosphate with the

enzyme apyrase, vesicle transport is inhibited and most vesicles are subject to a constrained brownian motion². Apparently, rigor complexes are not favoured in the vesicle-transport system in low ATP concentrations.

The structural organization of the vesicle transport system may be analogous in some respects to that of the actomyosin and the dynein-microtubule systems¹⁵⁻¹⁷. Significantly, in these three systems a crossbridge is the structural link between the stationary substrate and the translocated partner^{5,8,15}. However, although there may be structural analogies between these motile systems, the results presented here and others^{18,19} indicate that the enzymatic properties of the motile protein of vesicle transport differ significantly from the crossbridge proteins in both muscle and axonemes (that is, myosin and dynein, respectively).

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Primary structure of bovine thyroglobulin deduced from the sequence of its 8,431-base complementary DNA

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In mammals, an adequate supply of thyroid hormones is essential for normal growth and neurological development. The biosynthesis of thyroid hormones involves an iodinated precursor protein, thyroglobulin, which may be considered an extreme example of a pro-hormone¹. Thyroglobulin is a dimeric glycoprotein of relative molecular mass (M_r) 660,000 (660K), which is secreted by the thyrocyte and stored in the lumen of the thyroid follicle. The hormonogenic reaction is extracellular, and involves iodination of tyrosyl residues of thyroglobulin and the intramolecular coupling of a subset of these into thyroxine (T_4) and triiodothyronine (T_3), which remain part of the polypeptide chain. Secretion of hormones results from the endocytosis of thyroglobulin followed by its complete hydrolysis in lysosomes. Considering that the maximum yield of hormones is ~6-8 per 660K protein², the whole process is apparently wasteful. However, the efficiency of thyroglobulin as a thyroid hormone precursor is extremely high when the supply of iodine is short; in such conditions, almost all the iodine incorporated is found in iodothyronines³. Hence it is suggested that the thyroglobulin structure has evolved to allow for the preferential and efficient iodination and coupling of the hormonogenic tyrosines. Here we report the complete primary structure of bovine thyroglobulin, derived from the sequence of its 8,431-base-pair complementary DNA. The 2,769-amino-acid sequence is characterized by a pattern of imperfect repeats derived from three cysteine-rich motifs. Four hormonogenic tyrosines have been precisely localized near the amino and carboxyl ends of the protein.

The complete nucleotide sequence of bovine thyroglobulin messenger RNA (Fig. 1) was obtained by sequencing six recombinant plasmids containing 99% of its cDNA⁴ and one genomic

[illegible]

[illegible]

Fig. 1 Sequence of thyroglobulin cDNA and the deduced primary structure of the protein. Nucleotides are numbered (on the left of the figure) from the ATG initiation codon. The deduced amino acids are numbered from the amino-terminal asparagine of the mature protein. The polyadenylation signal⁸ is underlined. The putative *N*-glycosylation sites (Asn-X-Ser/Thr) and the tyrosine residues involved in hormonogenesis are underlined and overlined, respectively.

Methods. Six recombinant plasmids⁴ and one genomic DNA fragment (G. de Martynoff *et al.*, in preparation) were used as starting material. Subcloning in phage M13 vectors²⁴ and generation of overlapping fragments with DNase were performed as described in ref. 25. The dideoxy chain termination method²⁶ and buffer gradient gels²⁷ were used for DNA sequence analysis. Confidence was obtained by reading each nucleotide on both DNA strands. The overlapping recombinant plasmids⁴ differ at two positions: nucleotide 4,583 is A in pbTg 1.9 and G in pbTg 4.7 (changing amino-acid 1,510 from Ser to Gly), and nucleotide 7,141 is G in pbTg 4.7 and A in pbTg 2.5 (changing amino-acid 2,362 from Val to Met). These differences may reflect sequence polymorphism. However, an error in the reverse transcriptase reaction cannot be excluded.

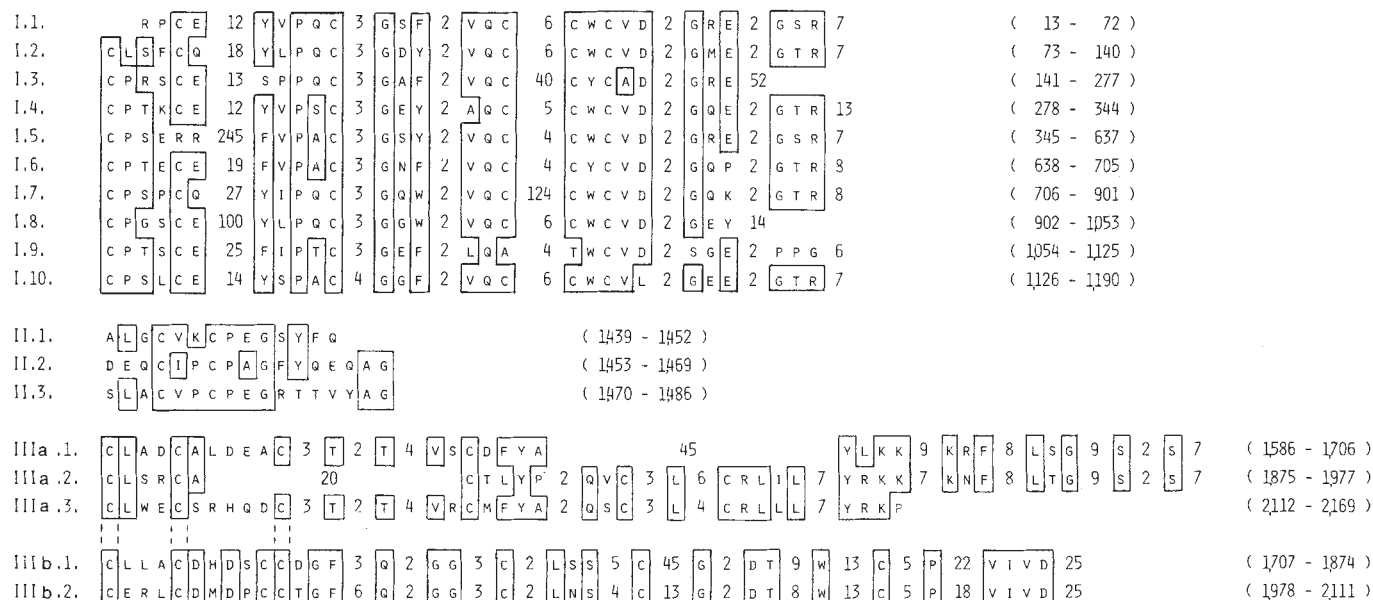


Fig. 2 Internal homology in thyroglobulin primary structure. The three types of homologous domains (given in the one-letter code) are aligned. Insertions or deletions have been introduced to maximize homology. Conserved amino acids are boxed according to the following rule: for type I repeats, the amino acids should be present at least five times; for type II and III repeats, at least twice. Residues showing no conservation between the domains are represented by numbers. Homologies in Cys positions between domains IIIa and IIIb are indicated by broken lines. Localization of the domains is shown on the right; the numbering is as in Fig. 1.

DNA fragment containing the 5'-terminal portion of the message (G. de Martynoff *et al.*, in preparation). Partial nucleotide sequences have been reported for three species⁵⁻⁷ but they do not exceed 3,000 bases long. The general organization of the mRNA sequence is as follows: a 41-nucleotide 5'-untranslated segment precedes an open reading frame of 8,307 bases. To our knowledge, this represents the longest coding sequence determined to date. The 3'-untranslated segment is 83 bases long and contains the canonical AATAAA polyadenylation signal⁸.

As judged from the nucleotide sequence, a 19-amino-acid signal peptide is followed by 2,750 residues, constituting a polypeptide of expected M_r 302,253; this value is in perfect agreement with the M_r obtained for the translation product of thyroglobulin mRNA *in vitro*⁹ or in *Xenopus* oocyte¹⁰ and with the M_r of 330,000 for the glycosylated protomer¹¹. The amino-acid composition of the protein agrees with published results (not shown). In particular, despite their role in hormonogenesis, the number of tyrosine residues (72, that is, 2.5%) does not deviate significantly from the average observed in a protein data bank (3.3%; ref. 12). The sequence contains 15 potential acceptor sites for *N*-glycosylation (Fig. 4), which represents a slight excess compared with the 20 chains characterized in the mature dimeric protein¹³.

When analysed for the presence of internal homology, the protein sequence shows a striking repetitive pattern involving three types of cysteine-containing motifs (Fig. 2). The type I motif is repeated 10 times between positions 13 and 1,190; it extends over ~60 amino acids and contains 24 highly conserved residues, including six cysteines. Sequences of variable length, unrelated to the motif, interrupt some of the repeats at fixed positions (Fig. 2). Preliminary data indicate that the positions of these insertions correlate with the borders of exons (J. Parma, in preparation). The type II motif is much shorter (17 amino acids) and is only repeated three times between positions 1,439 and 1,486. The type III motif may be subdivided into two subtypes (IIIa, IIIb; Fig. 2), which display a similar pattern of cysteine residues. Both subtypes are repeated twice in tandem, between positions 1,586 and 2,111. A third copy of motif IIIa is found further downstream (Fig. 4). Over 75% of the thyroglobulin sequence is thus involved in some kind of repetitive structure, clearly indicating that this exceptionally large protein evolved from the serial duplication of a limited number of building blocks. Interestingly, the last 600 residues at the carboxyl end show no internal homology, are poor in cysteine and contain a cluster of tyrosines of which three are hormonogenic (see below and Fig. 4).

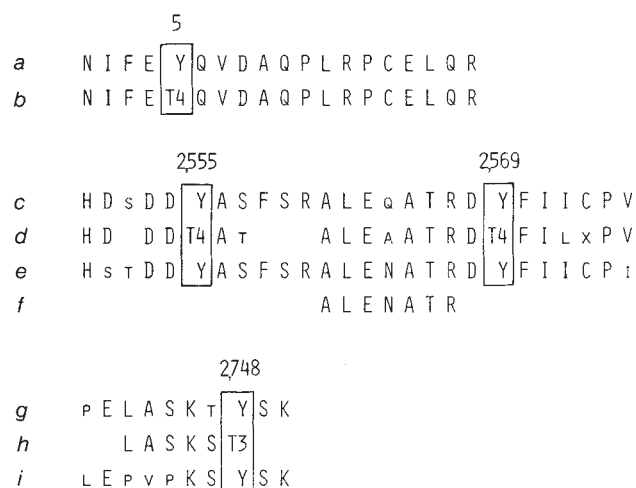


Fig. 3 Hormonogenic domains in thyroglobulin. The hormone-containing domains identified to date in various species are represented by the one-letter code and are aligned for maximum homology. The capital letters correspond to amino acids occurring at least twice in different species. The four hormonogenic tyrosines are boxed and their positions in the protein are indicated. *a*, *c*, *g*, the peptide sequences derived from the present study; *b*, the four identical hormone-containing peptides determined from a partial rat cDNA sequence⁷; *f*, a tryptic peptide, isolated from human thyroglobulin, containing a glycosidic chain attached to the asparagine residue³⁰; *h*, sequence of a hormone-containing peptide isolated from porcine thyroglobulin¹⁹.

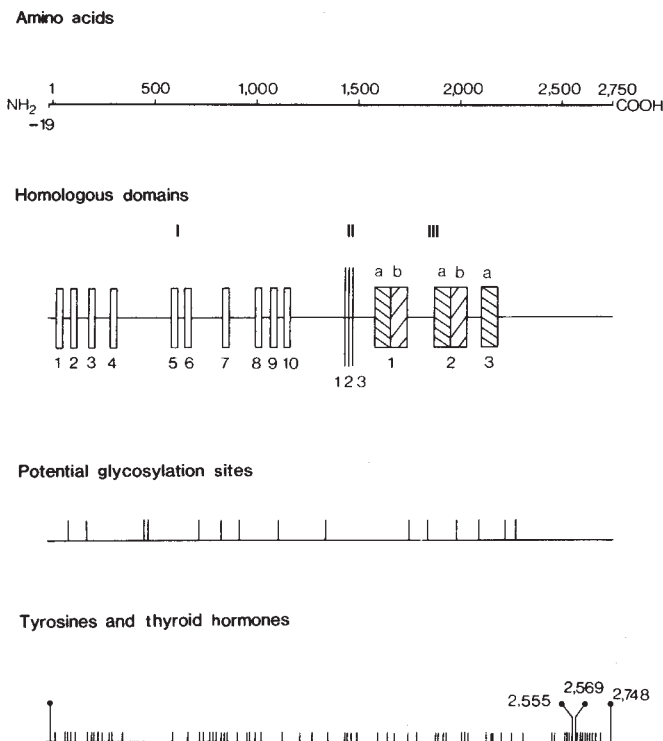


Fig. 4 Schematic representation of the thyroglobulin precursor. For clarity, a space has been introduced between the homologous domains even when they are contiguous in the protein (see Fig. 2). The tyrosine and thyroid hormone residues are represented by short and longer bars, respectively.

The sequence organization of thyroglobulin (Fig. 4) is reminiscent of the structure of some other large polypeptides, including the epidermal growth factor (EGF) precursor¹⁴, EGF receptor¹⁵ and low-density lipoprotein receptor¹⁶. Although there exists no direct sequence homology between thyroglobulin and these proteins, they probably share an analogous evolutionary history and may have similar three-dimensional structures.

Four tryptic peptides containing thyroid hormones have been isolated from thyroglobulin of various species and sequenced¹⁷⁻¹⁹. A systematic search for homologous peptides in our sequence allowed the identification and precise localization of all four hormonogenic tyrosines (Figs 3, 4). Two of these map at subterminal positions (residues 5 and 2,748); they correspond to sites involved preferentially in the synthesis of T₄ and T₃, respectively^{17,19}. The other two sites are closely linked around position 2,560. The amino-terminal domain is adjacent to the first, type I, repeat and might even share sequence characteristics with it⁵. In contrast, the three other hormonogenic tyrosines map in the non-repeated carboxy-terminal segment (Fig. 4). Comparison of the sequences surrounding the hormonogenic residues reveals no clear homology except for the presence of at least one charged amino acid (Fig. 3) and some clustering of tyrosines (Fig. 4)—the significance of this observation awaits the identification of the donor tyrosine involved in the coupling reaction.

Compilation of the sequences of the hormonogenic segments presently available shows a remarkable conservation of the amino-terminal domain (Fig. 3). It has been reported that as much as 50% of the thyroxine in mature thyroglobulin may originate from this domain²⁰. Although less information is available for the other hormonogenic domains, they do not display the same degree of sequence conservation (Fig. 3). Given these considerations, it is possible that the amino-terminal region of thyroglobulin has an important role in hormonogenesis. It is noteworthy, in this context, that a 10K amino-terminal peptide is cleaved from the protein during the hormonogenic process²¹.

The chemical or enzymatic reactions responsible for this phenomenon are unknown; it must be quite specific, however, as the carboxyl terminus of the 10K peptide could be unambiguously determined and was readily localized (Gln 80) in a partial protein sequence²².

Knowledge of the primary structure of thyroglobulin should provide the basis for studying the structure-function relationships of the hormonogenic process *in vitro*, in reconstructed synthetic systems, and should help to elucidate the molecular basis for genetic defects of thyroglobulin production²³.

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Ethylation interference and X-ray crystallography identify similar interactions between 434 repressor and operator

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In the crystal structure of a repressor-operator complex (the 434 repressor DNA-binding domain and its 14-base pair (bp) operator), Anderson *et al.*¹ elsewhere in this issue identify six positions of likely contact between repressor protein and phosphates of the DNA backbone. At each of these positions, electron densities of protein and DNA merge. Experiments presented here indicate that intact 434 repressor approaches these phosphates very closely when it is bound to DNA in solution. Specifically, when any one of these phosphates is ethylated², repressor cannot bind to the modified operator. We also identify another position where ethylation has

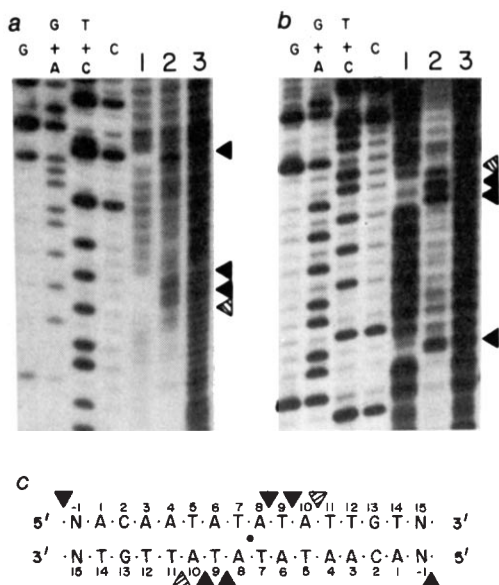


Fig. 1 Ethylation interference analysis of 434 repressor-operator interactions. *a*, Results for top strand; *b*, results for bottom strand. Lane 1, DNA bound by repressor; 2, DNA not bound by repressor; 3, ethylated unseparated control. In *a* and *b*, bands depleted in lane 1 and enriched in lane 2 relative to the control in lane 3 indicate ethylphosphates that interfere with repressor binding. *c*, Ethylphosphates that interfere with repressor binding aligned with the 434 operator sequence. Filled triangles, strong interference; hatched triangles, weaker interference.

Methods. A double-stranded consensus 2-fold-symmetric 434 operator of identical sequence to that used by Anderson *et al.*¹ with single-stranded *SalI* ends was generated by self-annealing of an oligonucleotide of sequence 5' TCGAACAATATATATTGT 3' (made on an Applied Biosystems Model 380A DNA synthesizer). This fragment was treated with T4 kinase (BRL) and ATP, then cloned using standard methods⁷ into the unique *SalI* site within the *lacZ* gene of pEMBL8- (ref. 8) to generate pFB37. Because insertion of the oligonucleotide destroys the *SalI* site, vectors without the insert in the ligation product could be linearized by digestion with *SalI* and hence eliminated during transformation. The 18-bp insertion preserves the *lacZ* translational reading frame by inserting 6 codons, none of which are translational stops. Plasmids with cloned operators could therefore be identified by transforming *Escherichia coli* strain JM101 (ref. 9) (phenotypically LacZ-), plating on X-Gal¹⁰ and picking blue Lac Z+ colonies. Plasmids containing single operators were identified by DNA sequencing¹¹ and cleaved at the unique *EcoRI* site. Cleaved plasmids were 3'-labelled (top strand) with DNA polymerase I large fragment (NEB) and [α -³²P]dATP (NEN), or 5'-labelled (bottom strand) by successive treatments with bacterial alkaline phosphatase (Boehringer) and T4 kinase and [γ -³²P]ATP (ICN; reactions as in ref. 7). After cleavage with *HindIII*, the labelled operator-containing fragment was isolated by electrophoresis on a 5% polyacrylamide gel, excision of the desired band and electroelution in 0.2× TAE buffer⁷. After filtration (Milex 0.45- μ m HA filters), phenol extraction, ether extraction and ethanol precipitation, DNA was ethylated with ethylnitrosourea (Sigma) as described². Ethylnitrosourea-treated DNA ($\sim 5 \times 10^{-8}$ M) was incubated at 4 °C with 2×10^{-7} M 434 repressor (isolated as described previously¹²) in 50 mM KCl, 2 mM MgCl₂, 10 mM Tris pH 7.4, 0.5 mM EDTA, 1 mM dithiothreitol, 50 μ g ml⁻¹ bovine serum albumin and 5% glycerol. Repressor-operator complexes were separated from unbound DNA that contained ethylations interfering with repressor binding by polyacrylamide gel electrophoresis³⁻⁵. Bound and unbound fractions were excised from the gel and purified as described above. Ethylated DNA fragments were cleaved at phosphotriesters by heating in alkali², prepared for electrophoresis as described³ and analysed on a 10% sequencing gel. Ethylation cleavage products were aligned with the sequencing ladder by correcting down to the nearest base (see ref. 2 for discussion). Alignment of a 5'-labelled sequencing band with a 5'-labelled ethylation cleavage product identifies the phosphate 5' to that base; alignment of a 3'-labelled sequencing band with a 3'-labelled ethylation cleavage product identifies the phosphate 3' to that base.

a significant but less dramatic effect on repressor binding, and note that in the structure, repressor closely approaches this phosphate. Our results strongly support the idea that the interactions between protein and the DNA phosphate backbone in the crystallized complex¹ are the same as those made by intact repressor to operator DNA in solution. In addition, our results suggest that DNA is slightly bent by repressor binding.

Phosphates of the 434 operator which, when ethylated, interfere with 434 repressor binding were identified by the method of Siebenlist and Gilbert² (modified as described by Hendrickson and Schleif³, see Fig. 1 legend). Briefly, the 2-fold-symmetric consensus 434 operator used by Anderson *et al.*¹ was cloned on a plasmid, end-labelled and ethylated such that each DNA molecule had an average of no more than one ethylphosphate. Purified ethylated fragment was incubated with excess 434 repressor, then operators bound to repressor were separated from the unbound operators by electrophoresis³⁻⁵. The unbound DNA molecules contain ethyl groups that prevent binding. Bound and unbound DNA fragments were isolated, cleaved at sites of ethylation and examined by electrophoresis on a polyacrylamide sequencing gel. Each band on the gel is generated by cleavage at a specific ethylphosphate. Bands enriched in the unbound fraction and depleted in the bound fraction indicate phosphates at which ethylation inhibits repressor binding.

Ethylation at any of three phosphates found 5' to bases -1, 9, and 10 on each strand prevents binding at the repressor concentration used here (compare lanes 1 and 2, Fig. 1*a, b*). Anderson *et al.*¹ show that these phosphates lie near residues Asn 16 and Gln 17 at the N-terminus of helix α 2 (phosphate -1); and Lys 40, Arg 41 and Arg 43 (phosphates 9 and 10). Ethylation of a fourth phosphate, 5' to base 11, has a significant but weaker effect on binding (hatched triangles in Fig. 1). In our interpretation of the structure, this phosphate is positioned within reasonable hydrogen-bonding distance of the γ -O of Ser 30. Thus, there is excellent agreement between the results of these chemical probe experiments and the co-crystal structure. The four phosphate contacts are symmetrically disposed about the centre of the operator (Fig. 1*c*) on one face of the B-DNA helix (Fig. 2).

Ethylation of a DNA phosphate removes the negative charge and adds a group with a van der Waals radius of ~ 4.5 Å. Both steric and charged-group effects can therefore contribute to its influence on binding. Model building with straight DNA shows that with the relationship of DNA and protein seen in the crystal, the side chains of Asn 16 and Gln 17, the only residues near



Fig. 2 Phosphates of the 434 operator at which ethylation reduces affinity for 434 repressor. Positions of strong ethylation interference are symbolized by filled circles and weaker interferences by hatched circles on a B-DNA helix. The operator centre of symmetry is indicated with a small filled circle.

the -1 phosphate, are too far from the DNA backbone for hydrogen-bond formation (Fig. 3a). If the DNA is bent to a radius of curvature of ~ 100 Å, then a hydrogen bond to the -1 phosphate can readily be made (Fig. 3b). Other small deformations could have a similar effect. An additional contribution to binding might come from favourable interaction between the partial positive charge on the N-terminus of helix $\alpha 2$ and the negative charge on the phosphate⁶. Based on the observed significance of the interaction at the -1 phosphate, we propose an explanation for some aspects of the crystal structure. In the co-crystal, 14-bp operators, each bound by a dimer of repressor DNA-binding domain, pack end-to-end to form rods. Each

14mer is tilted slightly and displaced laterally with respect to the next, so that the 3' terminal base on each strand does not stack on the adjacent operator. A repressor monomer bound to a given 14mer reaches over to an adjacent 14mer to make the -1 phosphate contact, and the DNA structure deviates from regular B-like conformation in this region. We imagine that the tilting and displacement of the 14mers, and possibly the local departure of the DNA backbone from canonical B-form, are consequences of crystallization using discrete fragments rather than continuous DNA. We believe that these effects occur in order to accommodate the -1 phosphate interaction, which in continuous DNA seems to require a gentle bend.

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Photodriven transmembrane charge separation and electron transfer by a carotenoporphyrin-quinone triad

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Photochemical transmembrane electron transfer processes are an integral part of natural photosynthetic solar energy conversion and are also central to the design of biomimetic energy conversion schemes¹⁻⁶. Here we report the synthesis and membrane-associated photoelectrochemical properties of carotenoporphyrin-quinone triad (I), a compound containing a photochemically active porphyrin and electron donor and acceptor moieties, and with the molecular architecture necessary to span a phospholipid bilayer. On excitation of compound I by visible light, charge is separated across a planar phospholipid bilayer membrane (BLM) in an intramolecular step; in the presence of suitable electron donor and acceptor species in the aqueous phases, a steady-state photocurrent is observed in an external circuit bridging the BLM. Artificial membranes containing I thus mimic key features of the photodriven transmembrane electron transfer processes characteristic of photosynthetic organisms.

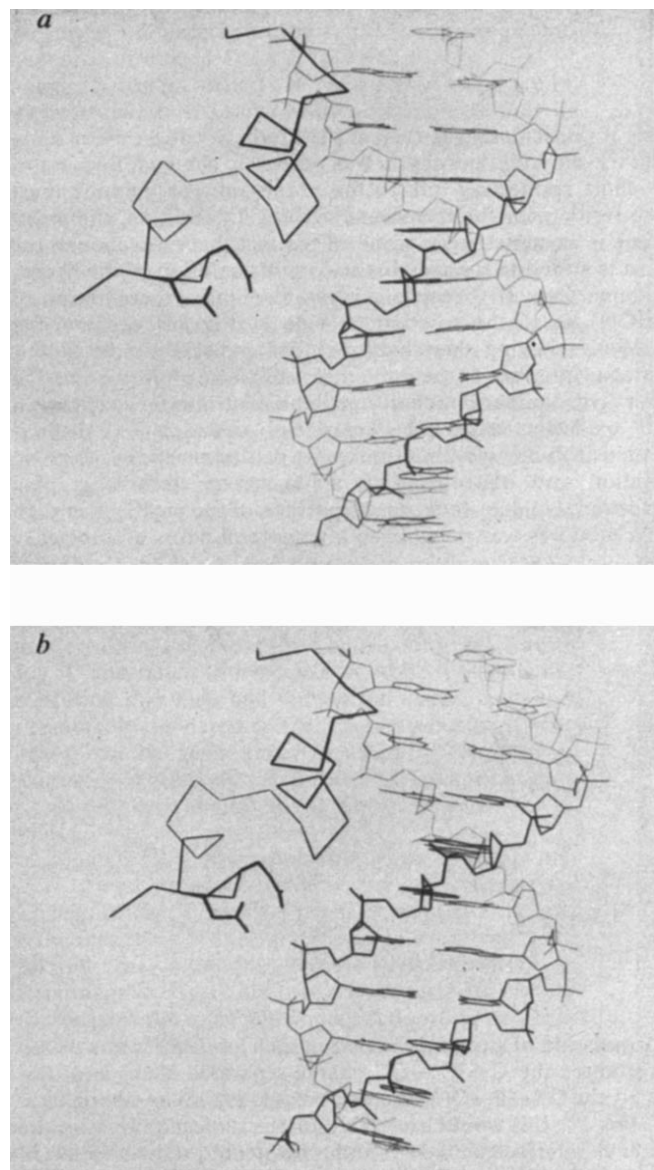


Fig. 3 Interaction of Asn 16 and Gln 17 with straight and bent DNA showing the α -carbon backbone of λ -repressor residues 30-60 (ref. 13), positioned as described by Anderson *et al.*¹, with the side chains of Asn 16 and Gln 17 of 434 repressor extending toward the DNA. In *a*, straight B-DNA was constructed by extending the double helix of the model DNA that best fits the electron density of one 14-bp operator¹. The amino nitrogens of the Asn 16 and Gln 17 side chains are both >5 Å from the oxygens of the -1 phosphate, which is too far for hydrogen-bond formation. In *b*, B-DNA with a slight right-handed supertwist, generated as described by Anderson *et al.*¹, was positioned so that 14 bp of the modelled DNA gave the best fit to the electron density of one 14-bp operator. The side-chain amino groups of Asn 16 and Gln 17 are separated from the phosphate oxygens by ~ 3 Å, a typical hydrogen-bond length.

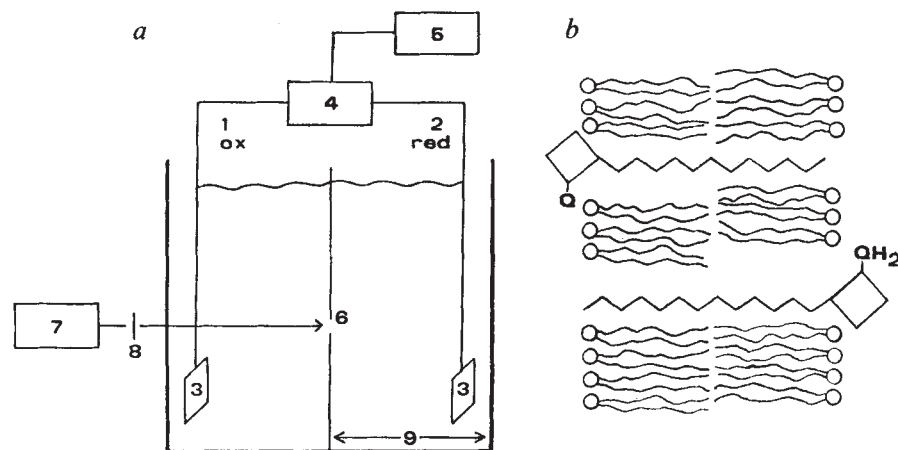


Fig. 1 *a*, Schematic diagram of a BLM cell for photoelectrical measurements: 1, side 1 containing oxidant ($\text{Fe}(\text{CN})_6\text{K}_3$), phosphate buffer and NaCl; 2, side 2 containing reductant (sodium ascorbate), phosphate buffer and NaCl; 3, Ag/AgCl electrode; 4, Keithley model 427 current-to-voltage converter; 5, Tektronix digital storage oscilloscope; 6, 1-mm-diameter orifice in thin Teflon divider; 7, laser; 8, shutter; 9, cell machined from block of Teflon. *b*, Schematic diagram showing the postulated arrangement of triads in the phospholipid bilayer. Note that triad I (labelled Q) is shown at the aqueous interface on side 1 and triad II (QH_2) at the interface on side 2.

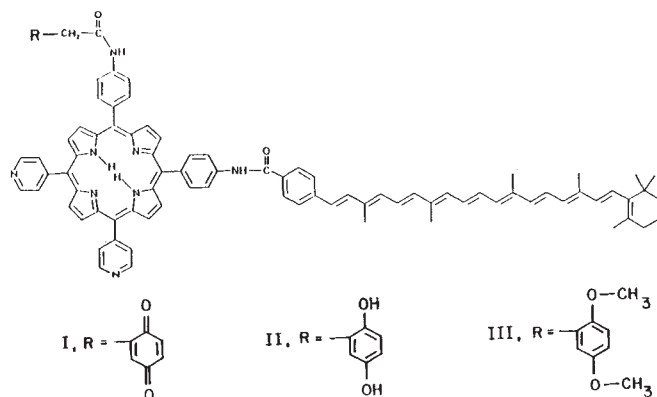
Photodriven charge separation in single-phase solution in compounds similar to I has been shown to be a two-step process involving electron transfer from the photoexcited porphyrin singlet state to the attached quinone, followed by electron transfer from the carotenoid to the porphyrin radical cation⁷⁻¹⁰. The resulting species, $\text{C}^{+}-\text{P}-\text{Q}^{-}$, is formed within ~ 100 ps of excitation and has a solvent-dependent lifetime on the micro-second timescale.

Triad I and its related forms II and III (see above) were synthesized by routes similar to those used previously^{7,11}. Their structures were verified using 500-MHz ^1H -NMR spectroscopy¹².

The photoelectrochemical experiments were carried out in the apparatus described previously¹³ (see Fig. 1*a*). The triad was dissolved in the appropriate membrane-forming solution (see Fig. 2 legend) and a small drop applied to the orifice in the Teflon divider. The phospholipid solution gradually thinned and formed a bilayer, as detected by coulometric measurements¹³. The current flowing through the cell, and therefore through the BLM, was detected as a function of time and displayed on a digital oscilloscope. In a typical experiment with triad I in the membrane, 1×10^{-2} M $\text{Fe}(\text{CN})_6\text{K}_3$ was dissolved in the aqueous phase on side 1 and 1×10^{-2} M ascorbic acid was placed on side 2. In the dark, no significant current was observed. However, irradiation of the BLM with 600-nm light from the laser produced an immediate photocurrent (Fig. 2). Typically (Fig. 2*a*), the photocurrent consisted of a transient component that rose with the opening time of the shutter (< 1 ms) and quickly decayed to a steady-state value (typically 50–500 pA). The peak of the transient current and the steady-state value varied considerably between samples. Generally, there was a strong positive correlation between the extent of bilayer formation, as estimated from the coulometric measurements, and the magnitude of the photocurrent. Moreover, on average, photocurrents obtained when the membrane-forming solution was prepared with hexadecane were larger than those when decane was used. This observation is consistent with the fact that solvents comprising hydrocarbon of longer chain length yield thinner membranes¹⁴. Figure 2*b* shows a photocurrent in which the transient component was not observed; such signals were less common than the first type, but occurred more frequently when the aqueous phases were at low pH and when the membranes were more than 10 min old and very thin (that is, when hexadecane was used as the solvent).

The currents discussed above are clearly the result of photo-induced electron transport across the phospholipid bilayer. We propose the following explanation for the results. The triad is a rod-like, amphipathic molecule that would be expected to lie in the bilayer as shown in Fig. 1*b*. The hydrocarbon-like carotenoid moiety (which extends ~ 39 Å from the porphyrin centre) should be well anchored in the low-dielectric membrane interior and should be aligned to some extent with the fatty-acid chains. The polar dipyrrolyl porphyrin moiety, on the other hand, should lie in the higher-dielectric region near the head groups.

This is particularly the case at pH 3, where one would assume that the dipyrrolyl porphyrin was positively charged. Because of its short connecting link to the porphyrin, the quinone must also reside near the membrane surface. In addition, the membrane is asymmetric by virtue of the fact that even though the triad is added to the membrane-forming solution in the hydroquinone form II (because it is more soluble), the addition of $\text{Fe}(\text{CN})_6\text{K}_3$ to the solution on side 1 (Fig. 1*a*) converts the molecules having their hydroquinone moieties at the side 1 aqueous interface to the quinone form. Those molecules having their hydroquinone moieties at the side 2 interface remain in that oxidation state. (This interfacial oxidation was demonstrated in bulk two-phase mixtures of the membrane-forming solution and aqueous ferricyanide, using chromatographic detection. No detectable decomposition of the pigments in such bulk mixtures was noted even after several hours of contact.)



Irradiation of a triad quinone I in such a bilayer is postulated to produce the $\text{C}-\text{P}^{+}-\text{Q}^{-}$ charge-separated state, and ultimately the $\text{C}^{+}-\text{P}-\text{Q}^{-}$ state, as observed in similar monophasic systems⁷⁻¹⁰; this would lead to a quinone radical anion near the aqueous interface on side 1, and a carotenoid radical cation in the interior of the bilayer. The quinone radical anion then donates an electron to ferricyanide to regenerate the neutral quinone, and the carotenoid radical cation, which can nearly span the bilayer, can accept an electron from the ascorbate on side 2 to regenerate the neutral carotenoid. The net result is the vectorial transfer of an electron from side 2 to side 1 and the regeneration of the triad photocatalyst. The charge separation across the membrane is countered by electron flow through the electrodes and detected as current in the external circuit.

There are several lines of evidence supporting the above interpretation of the experimental results. First, laser-flash photolysis of phosphatidylserine (or ethanolamine) liposomes containing compound I with 590-nm light resulted in a transient species having a maximum wavelength (λ_{max}) of 970 nm; the spectrum of this transient confirmed that it was indeed the

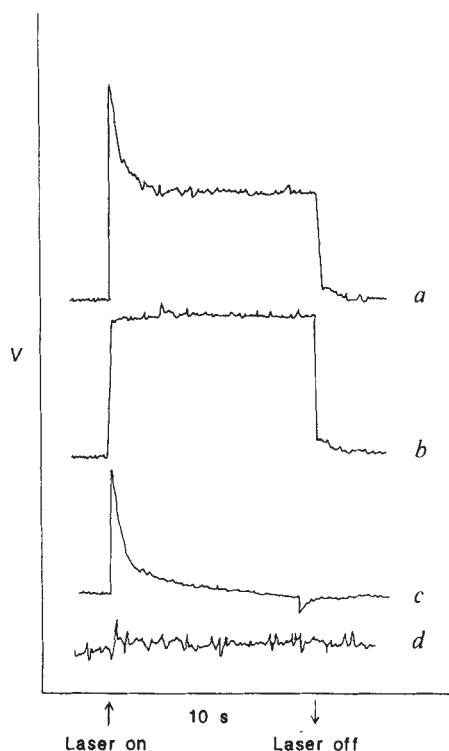


Fig. 2 Transmembrane photocurrents observed on irradiation of triads in BLM systems. *a*, pH 6.5, peak current 200 pA, 1×10^{-2} M ascorbate in side 2 and 1×10^{-2} M $\text{Fe}(\text{CN})_6\text{K}_3$ in side 1; *b*, pH 2.7, current 150 pA, same redox couples as in *a*; *c*, pH 5, peak current 29 pA, oxidizing couple as in *a*, no reductant in side 2, hexadecane as the solvent; *d*, triad III, noise peaks are ~ 5 pA, conditions as in *a*. The membrane-forming solutions used phosphatidylserine or phosphatidylethanolamine (3 mg), decane (100 μl) and ~ 0.05 mg of triad. In each case the membrane showed substantial thinning, as measured by coulometric response, greater than $5 \text{ G}\Omega$ d.c. resistance, and a dark current of < 5 pA. Each aqueous phase contained 0.1 M NaCl, 1×10^{-3} M phosphate buffer and a freshly prepared Ag/AgCl electrode. The sign of the observed currents is consistent with a negative charge carrier flowing from side 2 to side 1 through the bilayer. In these experiments there was no pH gradient (ΔpH) across the membrane. For *a*, *b* and *c*, triad II was used in the membrane-forming solution as described in the text.

carotenoid radical cation. Thus, the postulated charge separation does occur in the phospholipid bilayer. Furthermore, when the electron donor (ascorbate) was omitted from side 2, large transient photocurrents were observed on illumination, but a steady-state photocurrent was never detected (Fig. 2*c*). Similarly, no steady-state current was observed in the absence of ferricyanide. In addition, no transmembrane electrical response was detected for triad III in the conditions used for I

(Fig. 2*d*). In III, the hydroquinone moiety is protected as the dimethyl ether, and formation of the intramolecular charge-separated state is precluded. These three control experiments confirm the involvement of the transmembrane charge-separated state of triad I and the aqueous-phase redox couples in the observed steady-state photocurrents.

A final piece of evidence concerns the turnover number of the triad in the BLM. If the proposed scheme is indeed correct, then each triad I molecule should be capable of transporting many electrons across the bilayer. At the excitation wavelength of 600 nm, the porphyrin absorption cross-section is $\sim 1.7 \times 10^{-17} \text{ cm}^2$ per molecule and the laser intensity was $\sim 3 \times 10^{18}$ quanta per s cm^2 , yielding ~ 50 excitations per s molecule. From the composition of the membrane-forming solution, there are an estimated 10^{10} molecules per BLM and therefore 5×10^{11} excitations per s in the BLM. At a quantum yield of $\sim 10\%$ (refs 7–10), this corresponds to a photocurrent of ~ 4 nA, a value recorded in some of the BLM experiments. Although very approximate, this result is certainly consistent with a steady-state process having a high turnover number and capable of operating for many seconds.

Note that this example of transmembrane electron transfer is essentially the light-driven catalysis of the thermodynamically spontaneous oxidation of ascorbate by ferricyanide ion. None of the intramolecular redox potential of the charge-separated state $\text{C}^{+}\text{--P--Q}^{-}$ is conserved as transmembrane chemical potential. However, photocurrents opposing the applied electrical potential were observed even when side 1 was 100 mV negative to side 2. The judicious choice of redox couples and a properly asymmetrized membrane should ultimately make it possible to achieve net energy conservation in this system.

In photosynthetic membranes, carotenoids prevent the sensitization of oxygen by chlorophyll triplet-state species and thereby perform a photoprotective function^{15,16}. These carotenoporphyrin triads have sufficient electronic coupling between the carotene and porphyrin moieties¹¹, via the amide linkage, to mediate extremely rapid triplet-triplet energy transfer ($\text{C}^{+}\text{P--Q} \rightarrow {}^3\text{C--P--Q}$)⁹. Therefore, even though excitation of the hydroquinone triads results in a high yield of porphyrin triplet, energy transfer to the attached carotenoid occurs on the nanosecond timescale and precludes singlet oxygen formation. These artificial membranes are thus photoprotected in precisely the same way as native photosynthetic membranes.

The photodriven charge separation by carotenoporphyrin-quinone triads across artificial membranes, as demonstrated here, serves as a paradigm for many transmembrane electrochemical events in energy-transducing biological membranes, and will allow detailed investigations of photodriven charge separation across both chemical and electrical potentials, which should in turn lead to a better understanding of photosynthetic energy conversion.

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Hydrogen bonding in enzymatic catalysis analysed by protein engineering

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Two historic publications have suggested that enzymes can utilize the binding energy with their substrates to increase the rate of chemical catalysis—a concept termed strain^{1,2}. Specifically, the structure of an enzyme was described as complementary to the structure of the transition state of its substrate rather than to that of the unreacted substrate, so that the substrate would tend to be distorted on binding to the enzyme. The current understanding of strain does not require such distortion. Instead, it is thought that there are groups on the enzyme which interact better with the transition state than with the unaltered substrate, an arrangement known as differential binding of substrates and transition states³. The improvement in binding energy caused by going from substrate to transition state lowers the activation energy of the reaction and so increases the catalytic rate. Here we report direct evidence in support of this notion. Rapid reaction kinetics on mutants of a tyrosyl-transfer RNA synthetase produced by protein engineering show that certain hydrogen-bonding side chains some distance from the reacting bonds of the substrate bind to ATP preferentially in the transition state of the reaction rather than in the unreacted state. In this way, the binding energies of the interactions are used primarily to lower the apparent activation energy of the chemical steps rather than to enhance binding.

The hypothesis of better binding in the transition state has been tested using substrates and inhibitors with varying structures. Two lines of supporting evidence have been adduced from classical enzyme kinetics³. First, transition-state analogues, which usually mimic the changes in geometry about the bonds that are being broken and made in the reaction, often bind between 10² and 10⁴ times more strongly than the original substrates. Second, experiments on proteases with broad specificity show that additional groups on a substrate can lead to an increase in k_{cat} rather than stronger binding. These experiments suggest that groups on the substrate that are far from the seat of reaction can alter the catalytic rate. Many of the substrates

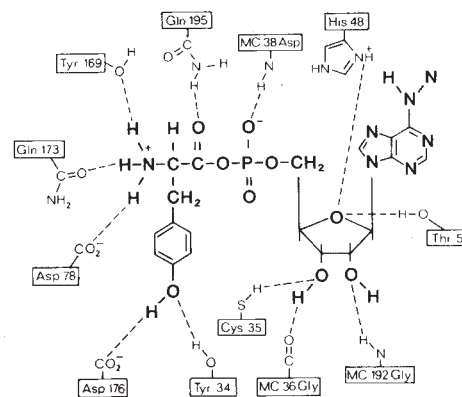
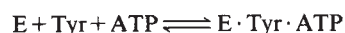


Fig. 1 Side chains of the tyrosyl-tRNA synthetase that form hydrogen bonds with tyrosyl adenylate (courtesy of D.M. Blow and P. Brick).

used in these studies, however, differ greatly from each other or from the specific substrates, and the results may arise simply from the nonproductive binding of the smaller substrates³.

The advent of protein engineering allows the idea of differential binding of transition states and substrates to be tested directly by varying the structure of the enzyme. An amino-acid side chain that interacts with the substrate can be altered to remove the interaction. If the side chain binds equally well with the substrate in both the ground and transition states, then removal should raise the dissociation constant K_S and not affect k_{cat} . Conversely, if the side chain binds the substrate only in the transition state, then removal should leave K_S unaltered and just lower the value of k_{cat} . Importantly, just one interaction out of many with the substrate may be changed at a time so that artefacts associated with a change of mode of binding of the substrate will be minimized. The contributions of individual interactions to differential binding energies are not known and this approach enables them to be quantified.

Genes of mutant tyrosyl-tRNA synthetases (from *Bacillus stearothermophilus*), which have had the relevant hydrogen-bonding groups removed, have been generated for use in experiments to determine the role of hydrogen bonding in biological specificity⁴. Values of k_{cat} and K_M for tyrosine and ATP for the activation reaction (equation (1)) were measured by pyrophosphate



exchange. Unfortunately, because of the complex kinetics, the values of k_{cat} and K_M for pyrophosphate exchange are composite

Table 1 Activation of tyrosine by tyrosyl-tRNA synthetases

Enzyme	k_3 (s ⁻¹)	K'_S (ATP) (mM)	K_S (Tyr) (μM)
Wild type	38	4.7	12
Δ(321-419)*	34	5.2	12
Tyrosine binding-site mutants			
Tyr → Phe 34	35	4.4	29
Tyr → Phe 169*	35	4.6	1,320
ATP binding-site mutants			
Cys → Ser 35	4.7	4.8	8
Cys → Gly 35	4.0	4.5	11
His → Gly 48	9.9	9.9	23‡
Thr → Ala 51	75	4.7	12

Experiments performed at 25 °C at pH 7.78 (144 mM Tris-HCl) in the presence of 10 mM free MgCl₂. k_3 , Rate constant for the formation of E.Tyr-AMP obtained by stopped-flow fluorimetry⁸, with standard error ±5%; K'_S (ATP) dissociation constant of ATP from the E.Tyr.ATP complex, determined as for k_3 ; K_S (Tyr), dissociation constant of Tyr from the E.Tyr complex obtained from equilibrium dialysis⁸.

* Truncated enzyme lacking the tRNA binding domain (residues 320-419)⁹.

† Mutation on truncated enzyme with the reference enzyme being the truncated wild-type enzyme.

‡ This value is abnormally high and may be caused by a loss of an electrostatic interaction with His 48.

Table 2 Apparent binding energies of side chains to substrates and to transition states

Side chain	Binding energy with substrate* (kcal mol ⁻¹)	Binding energy with transition state† (kcal mol ⁻¹)
Tyrosine binding site		
Tyr 34 —OH	-0.52	-0.57
Tyr 169 —OH	-2.58	-2.58
ATP binding site		
Cys 35 —SH	-0.03	-1.21
His 48 —imidazole	-0.44	-1.24
Thr 51 —OH	0.00	+0.40

* Calculated from $\Delta G = -RT \ln K_{mut}/K_{wt}$, where K is the relevant dissociation constant of the substrate and mut and wt refer to mutant and wild-type enzymes respectively.

† Calculated from $\Delta G = RT \ln (k_3/K)_{mut}/(k_3/K)_{wt}$.

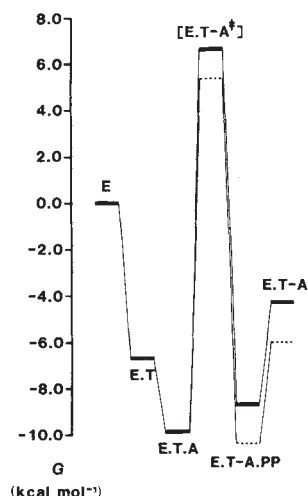
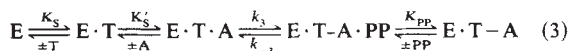
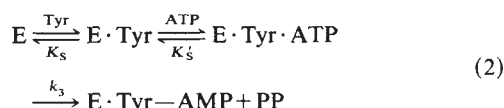


Fig. 2 Gibbs free-energy profiles for the formation of tyrosyl adenylate (E.T-A) by wild-type enzyme (---) and the mutant Cys → Gly 35 (—) (equation (3)). The rate constants for the formation of E.T-A and the transition state E.T-A* are taken from Table 1.



The reverse rate constant k_{-3} (16 s^{-1} for wild type, 33 s^{-1} for mutant) and dissociation constant for pyrophosphate, K_{PP} (0.61 mM for wild type, 0.63 mM for mutant) were measured by stopped-flow fluorimetry⁷. Free energies were calculated from the standard equations using a standard state of 1 M for tyrosine (T), ATP (A) and pyrophosphate.

values containing rate and equilibrium constants for the pyrophosphorolysis reaction. These data thus gave no conclusive evidence on the interconversion of binding and chemical activation energies. Values for k_3 and the dissociation constants (K_S and K'_S) in equation (2) can, however, be measured



directly by stopped-flow fluorimetry⁵ and equilibrium dialysis (Table 1). From these values, free-energy contributions to binding and catalysis for individual groups can be calculated using the standard equations³ (Table 2).

The residues that were mutated are all in the binding site of the enzyme, but are not directly involved in bond making and breaking (Fig. 1). Removal of side chains that bind to tyrosine (Tyr → Phe 34 and Tyr → Phe 169) does not significantly alter k_3 but increases the dissociation constant of tyrosine from the $E \cdot T \cdot A$ complex (Table 1). The hydroxyl moieties of Tyr 34 and Tyr 169, therefore, can be assumed to interact equally well with tyrosine in its unreacted form and in the transition state of the reaction. The binding energies of the -OH groups of Tyr 34 and Tyr 169 with the substrate and transition state are readily calculated³ and are seen to be nearly identical (Table 2).

In contrast, removal of side chains that interact with ATP causes significant changes in k_3 but, apart from His → Gly 48, hardly affects the value of K'_S for ATP. Mutation of Cys 35 to Gly 35 or Ser 35 leads to a 10-fold lowering of k_3 with no significant change in K'_S . Changing His → Gly 48 results in a mixture of effects, including a fourfold lowering of k_3 and a twofold increase in K'_S . Mutation of Thr → Ala 51 leads to a twofold increase in k_3 with no discernible effect on K'_S for ATP. This result is entirely consistent with the earlier proposal from structural observations and steady-state kinetic measurements that the bond between Thr 51 and the ribose ring oxygen is long and weak and contributes no binding energy^{5,6}. As the value of k_3 is increased on deletion of this bond, it must be even longer and weaker in the transition state. (The presence of an alanine at position 51 gives a more active enzyme, and it is intriguing

that the tyrosyl-tRNA synthetase from *Bacillus caldopenax*, which is 99% homologous with that from *Bacillus stearothermophilus*, does have alanine at position 51; ref. 7).

Thus, the side chains of residues 35, 48 and 51 probably have different interaction energies with ATP depending on whether it is in its unreacted form or its transition-state structure. The differences could arise from two effects, or a mixture of the two: (1) an unfavourable interaction in the enzyme-substrate complex that is relieved on formation of the transition state (substrate destabilization); and (2) an interaction that is not properly made in the enzyme-substrate but is realized in the enzyme-transition state complex (transition-state stabilization). We have shown by construction of the full free-energy profile for the formation of tyrosyl adenylate (Fig. 2) that the side chain of Cys 35 does not function purely by substrate destabilization. Removal of the side chain of Cys 35 (Cys → Gly 35) lowers the affinity of the enzyme for tyrosyl adenylate by the full amount expected for the loss of a hydrogen bond⁴, showing that it makes favourable interactions with the adenosine moiety of the products as well as with the transition state.

It seems unlikely from model building that the mutation of residues in this study will cause any structural artefact. Further, the double-mutant test has been applied to residues 35 and 48 and no change of structure was detected⁶.

We have concluded, therefore, that the interactions of important side chains of the enzyme with tyrosine remain unaltered but that those with ATP are optimized as the enzyme-substrate complex reaches the transition state. Thus we have shown that the binding energy of the enzyme and substrate can be used to enhance the catalytic rate and we have quantified its importance.

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Corrigenda

The nucleotide sequences of *copia* and *copia*-related RNA in *Drosophila* virus-like particles

T. Emori, T. Shiba, S. Kanaya, S. Inouye, S. Yuki & K. Saigo
Nature **315**, 773-776 (1985)

ALL U3s and U5s on page 776, lines 16-17 and in Fig. 2 should be U5s and U3s respectively.

Analytical solution for the effect of increasing CO₂ on global mean temperature

T. M. L. Wigley & M. E. Schlesinger
Nature **315**, 649-652 (1985)

IN Fig. 1 legend, the lower part of the figure should be that for $\kappa = 3 \text{ cm}^2 \text{ s}^{-1}$ and the upper part for $\kappa = 1 \text{ cm}^2 \text{ s}^{-1}$ and not vice versa. In the acknowledgements the NSF/DOE grant number should be ATM 8205-992.

Erratum

X-linkage of steroid sulphatase in the mouse is evidence for a functional Y-linked allele

E. Keitges, M. Rivest, M. Siniscalco & S. M. Gartler
Nature **315**, 226-227 (1985)

The first line of the first column on p. 227 was printed in the wrong position and should be read as the last line of the first column of that page.

Aromaticity of soil fulvic acid

FARMER and Pisaniello¹ claim that: (1) aliphatic and aromatic oxidation products of the podzol Bh fulvic acid, especially after premethylation with diazomethane, are mainly artefacts or contaminants; and (2) the formation of these oxidation products cannot be reconciled with the ¹³C-NMR spectrum of the fulvic acid.

I have the following comments: (1) Benzenecarboxylic, phenolic and aliphatic carboxylic acids are not only the major oxidation products of the alkaline KMnO₄ oxidation of unmethylated and methylated fulvic acid², but also result from the oxidation of unmethylated fulvic acid with alkaline CuO (ref. 3) and alkaline H₂O₂ (ref. 4) as well as HNO₃ (ref. 5) and CH₃CO₂OH (ref. 6). Thus, diazomethane has nothing to do with the types of products resulting from the oxidation of the fulvic acid.

(2) Both solid-state⁷ and liquid-state (refs 8, 9) ¹³C-NMR spectra of fulvic acid show signals near 20 and 30 p.p.m., indicative of the presence of terminal CH₃ and (CH₂)_n in alkyl chains. Other resonances are detected between 50 and 85 p.p.m., arising from amino acids and carbohydrates. Major signals near 130 p.p.m. indicate the presence of aromatic carbons not substituted by O or N. But in the liquid-state ¹³C-NMR spectrum of the methylated fulvic acid⁸, now soluble in CDCl₃, the broad band near 130 p.p.m. is resolved into four sharp signals, clearly indicating improved resolution. The comments of Farmer and Pisaniello on this point are irrelevant because they erroneously assumed that this spectrum was run in base instead of CDCl₃. Other strong signals in the ¹³C-NMR spectrum of fulvic acid appear near 180 p.p.m., due to carbon in CO₂H groups. While some humic substances show signals between 150 and 160 p.p.m., others do not. It is possible that because of substitution effects, the chemical shift due to phenolic carbon is moved downfield so that signals arising from phenolic carbon and carbon of CO₂H groups overlap. It is noteworthy that after methylation of the fulvic acid, the presence of phenolic carbon is indicated by a sharp signal at 56.3 p.p.m. Thus, contrary to the claims of Farmer and Pisaniello¹, the structural information on fulvic acid provided by ¹³C-NMR agrees with that indicated by chemical degradation.

Dialkyl phthalates are the only plasticizers that have so far been detected in oxidation products of fulvic acid². The oxidation of dialkyl phthalates would be expected to yield benzenedicarboxylic acids. But how can dialkyl phthalates be oxidized to benzenetri-, tetra-, penta- and hexacarboxylic acids and phenolic acids?

Therefore, it is highly unlikely that significant amounts of oxidation products arise from contaminants. Another important indicator of aromatic fulvic acid components is the relatively high content of free radicals⁴ that have the spectroscopic characteristics of semi-quinones. I conclude from the above that aromatic structures are significant constituents of fulvic acid. The question of whether or to what extent hydroxyl-substituted unsaturated aliphatic carboxylic acids are present in fulvic acid, awaits the isolation of significant amounts of such structures, which, so far, nobody has been able to do.

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FARMER REPLIES—Preston and Schnitzer¹ suggested that the four sharp aromatic carbon signals that they observed in the ¹³C-NMR spectrum of methylated fulvic acid might arise from benzene polycarboxylic esters, and supposed that the parent acids would not be detectable in ¹³C-NMR spectra of fulvic acid dissolved in alkali. We found² that such acids were readily detectable as sharp signals when added to a NaOD solution of fulvic acid: our comments are therefore entirely relevant, as they negate Preston and Schnitzer's hypothesis¹, and establish, conclusively, that simple benzene polycarboxylic acids are not even a minor component of fulvic acid. The four sharp signals observed in the methylated fulvic acid can arise from little more than four different types of benzenoid carbon. They are therefore unlikely to be associated with benzenoid carbons of the diverse types indicated by the multiple aromatic degradation products of fulvic acid, reported by Schnitzer and co-workers. The four bands are more likely to arise from a contaminant.

The references cited above by Schnitzer illustrate that reports of high yields of benzene and hydroxybenzene polycarboxylic acids in the oxidation products of humic and fulvic acids originate almost entirely from his laboratories. Consider-

ing, for example, only the non-phenolic benzene polycarboxylic acids, Schnitzer and co-workers have reported yields mostly in the range 6-14% of the starting materials. In contrast, Ogner³ and Spiteller⁴ obtained yields of only 0.12-0.38% of these acids. In a careful quantitative study using permanganate oxidation, H. A. Anderson of this institute (personal communication) has obtained a yield of 1.63% from a podzol Bh fulvic acid (Contech), similar to the Armadale preparation intensively studied by Schnitzer and co-workers. This yield does not greatly exceed that given by polymaleic acid in the same conditions (0.8%).

Following the suggestion² that ¹³C-NMR spectra could be used to estimate the amounts of benzene and hydroxybenzene polycarboxylic acids in the unfractionated oxidation products of fulvic acids, we have applied this technique to the products of peracetic acid oxidation. The results showed clearly that benzene polycarboxylic acids cannot be present in the amounts reported by Schnitzer and Skinner⁵.

In the absence of confirmation from other laboratories, the high yields of aromatic degradation products reported by Schnitzer and co-workers can carry no weight. We can only speculate on the reasons for these anomalously high yields.

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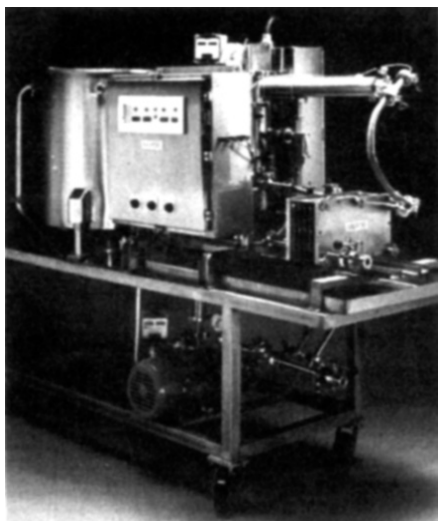
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The new products covered here are from some of the manufacturers exhibiting at the 13th International Congress of Biochemistry in Amsterdam.

• Labsystems UK has recently introduced two new instruments, the FP9000 Analyser for automatic batch processing, random access and profile chemistries and the Haematology Analyser for coagulation work. LabSystem's other products include ELISA kits for *Toxoplasma gondii*, cytomegalovirus, rubella, *Chlamydia*, *Bordetella pertussis* and tetanus toxoid, plus the Fecatest, a human Hb specific ELISA for the detection of colorectal carcinoma.

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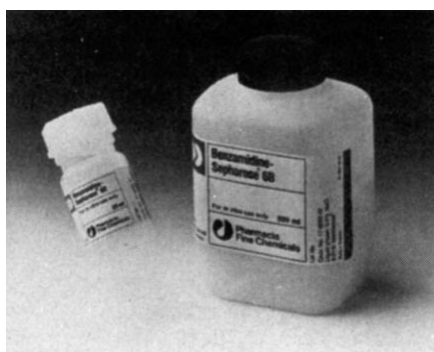
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Fsp I	5'...TCGCA...3'	Spe I	5'...ACTAGT...3'
Hin P I	5'...GCG...3'	Ssp I	5'...AATATT...3'
Nhe I	5'...GCTAGC...3'	Sty I	5'...CCAA...3'
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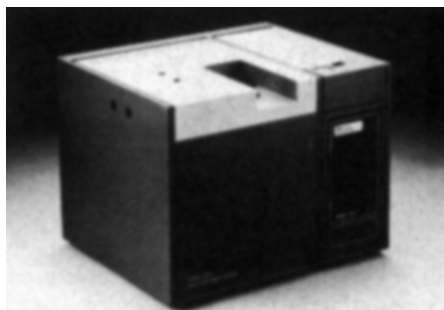
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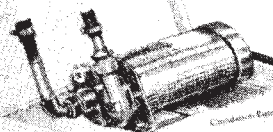
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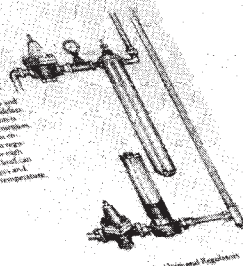
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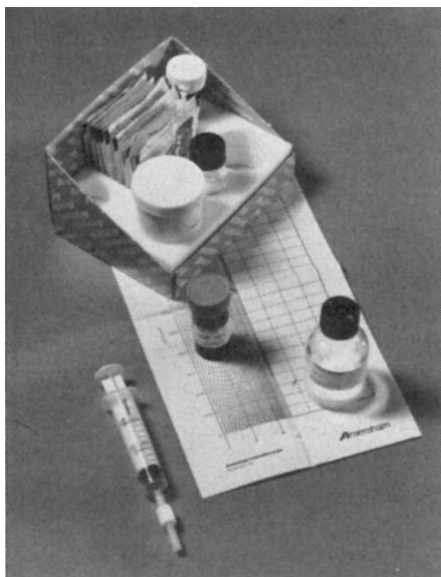
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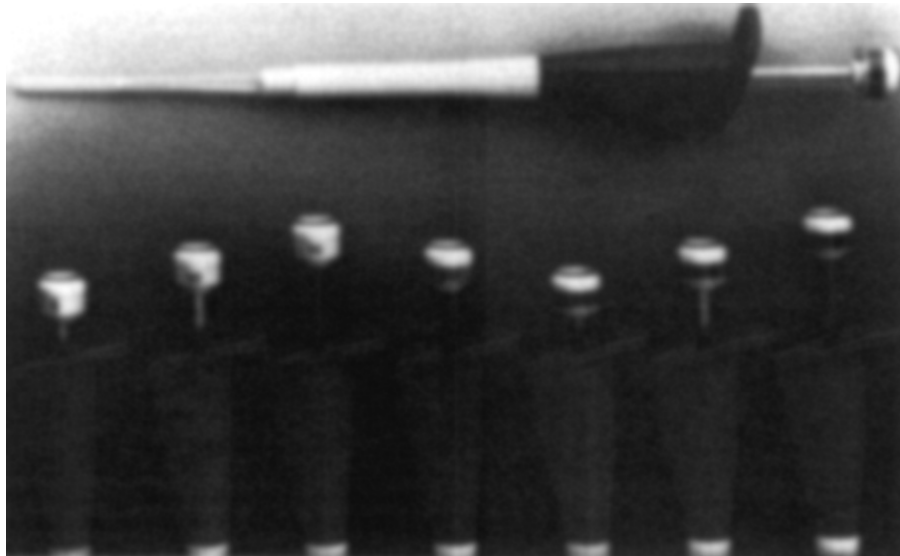
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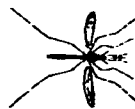
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